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METHODS AND COMPOSITIONS FOR THE PRODUCTION OF STABLY TRANSFORMED, FERTILE MONOCOT PLANTS AND CELLS THEREOF

DEKALB PLANT GENETICS
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Abstract: This invention relates to a reproducible system for the production of stable, genetically transformed maize cells, and to methods of selecting cells that have been transformed. One method of selection disclosed employs the Streptomyces bar gene introduced by microprojectile bombardment into embryogenic maize cells which were grown in suspension cultures, followed by exposure to the herbicide bialaphos. The methods of achieving stable transformation disclosed herein include tissue culture methods and media, methods for the bombardment of recipient cells with the desired transforming DNA, and methods of growing fertile plants from the transformed cells. This invention also relates to the transformed cells and seeds and to the fertile plants grown from the transformed cells and to their pollen.

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Detailed Description

METHODS AND COMPOSITIONS FOR THE PRODUCTION OF STABLY TRANSFORMED, FERTILE MONOCOT PLANTS AND CELLS THEREOF

FIELD OF THE IWENTIO The present invention relates to reproducible systems for genetically transforming monocotyledonous plants such as maize, to methods of selecting stable genetic transformants from suspensions of transformed cells, and to methods of producing fertile plants from the transformed cells. Ex emp lary transformation methods include the use of microprojectile bombardment to introduce nucleic acids into cells, and selectable and/or screenable marker systems, for example, genes which confer resistance (e.g., antibiotic, herbicide, etc.), or

- which contain an otherwise phenotypically observable trait. In other aspects, the invention relates to the production of stably transformed and fertile monocot plants, gametes and offspring from the transgenic plants.

DESCRIPTION OF THE RELATED ART

Ever since the human species emerged from the hunting-gathering phase of its existence, and entered an agricultural phase, a major goal of human ingenuity and invention has been to improve crop yield and to alter and improve the characteristics of plants. In particular, man has sought to alter the characteristics of plants to make them more tasty and/or nutritious, to produce increased crop yield or render plants more adaptable to specific environments.

Up until recent times, crop and plant improvements depended on selective breeding of plants with desirable characteristics. Initial breeding success was probably accidental, resulting from observation of a plant with desirable characteristics, and use of that plant to propagate the next generation. However, because such plants had within them heterogenous genetic complements, it was unlikely Mat progeny identical to the parent(s) with the desirable traits would emerge. Nonetheless, advances in controlled breeding have resulted from both increasing knowledge of We mechanisms operative in hereditary transmission, and by empirical observations of results of making varioustparental plant crosses.

Recent advances in molecular biology have dramatically expandid man I s ability to manipulate the germplasm of animals and plants. Genes controlling specific pheno typ es, for ex amp le specific polypeptides that lend antibiotic or herbicide resistance, have been located within certain germplasm and isolated from it.

Even more important has been Me ability to take the genes which have been isolated from one organism and to introduce them it" another organism. This transformation may be accomplished even "ere We recipient o0anism is from a different phylum, gems or species frWithat which donated the gene (heterologous transformation).

Attempts-have been made to genetically engineer desired traits into plant genomes by introduction of exogenous gyes using genetic engineering techniques.

These techhiques.have been successfully applied in some plant systm,-nincipally in dicotyledonous species.

The uptake ofnew DNA by recipient plant cells has been accomplished various means, including A"acterium infection (32), polyethylene glycol (PEG)-mediated DNA uptake (25)V electroporation of protoplasts (17) and micropm ~le bombardment (23). Unfortunately, the introduction of exogenous DM into monocotyledonous species and subsequent regeneration of transformed plants has proven much more difficult than transformation and regeneration in dicotyledonous plants. Moreover, reports of

methods for the transformation of monocotyledons such as maize, and subsequent production of fertile maize plants, have not been forthcoming. Consequently, success has not been achieved in this area and commercial implementation of transformation by production of fertile transgenic plants has not been achieved. This failure has been particularly unfortunate in the case of maize, "ere there is a particularly great need for methods for improving genetic characteristics.

Problems in the development of genetically transformed monocotyledonous species have arisen in a variety of general areas. For example, there is generally a lack of methods which allow one to introduce nucleic acids into cells and yet permit efficient cell culture and eventual regeneration of fertile plants.

only limited successes have been noted. In rice, for example, D% transfer has only recently been reported using protoplast electroporation and subsequent regeneration of transgenic plants (41). Furthermore, in maize, transformation using protoplast electroporation has also been reported (see, e.g., 17). However, recovery of stably transformed plants has not been reproducible. A particularly serious failure is that the few transgenic plants produced in the case of maize have not been fertile (38). Mile regeneration of A fertile corn plants from protoplasts has been reported (37, 39), these reported methods have been limited to the o use of non-transformed protoplasts. Moreover, regeneration of plants from protoplasts is a technique which carries its own set of significant drawbacks.

Even with vigorous attempts to achieve fertile, transformed maize plants, reports of success in this regard have not been forthcoming.

A transformation technique that circumvents the need to use protoplasts is microprojectile bombardment.

Although transient expression of a reporter gene was detected in bombarded tobacco pollen (47), stable transformation 0 microprojectile bombardment of pollen has not been reported for any plant species. Bombardment of soybean apical meristems with DM-coated gold particles.resulted'in chimeric plants containing transgenic sectors. Progeny containing the introduced gene were obtained at a low frequency (27). Bombardment of shoot meristems of immature maize embryos resulted in sectors of tissue expressing a visible marker, anthocyanin, Me synthesis of which was triggered by the introduction of a regulatory gene (46). An analysis of cell lineage patterns in maize (28) suggests that germline transformation of maize by such an approach may be difficult.

A second major problem in achieving successful monocot transformation has resulted from the lack of efficient marker gene systems which have been employed to identify stably transformed cells. Marker gene systems are those which allow the selection of, and/or screening for, expression products of DM. For use as assays for transformed cells, the selectable or screenable products should be those from genetic constructs introduced into Me recipient cells. Hence, such marker genes can be used to identify stable transformants.

Of We more commonly used marker gene systems are gene systems-which confer resistance to aminoglycosides such as kanamycin. While kan amy cin resistance has been used successfully-in both rice (51) and corn protoplast systems (A), it remains a very difficult selective agent to use in monocots due to hie endogenous resistance (19). Many monocot species, maize, in particular, possess high endogenous levels of resistance to aminoglycosides. Consequently, this class of compounds cannot be used reproducibly to distinguish transformed from non-transformed tissue. New methods for reproducible selection of or screening for transformed plant cells are therefore needed.

Accordingly, it is clear that improved methods and/or approaches to the genetic transformation of monocotyledonous species would represent a great advance in the art. Furthermore, it would be of particular significance to provide novel approaches to monocot transformation, such as transformation of maize cells, which would allow for the production of stably transformed, fertile corn plants and

progeny into which desired exogenous genes have been introduced.

Furthermore, the identification of marker gene systems applicable to monocot systems such as maize would provide a useful means for applying such techniques generally.

Thus, the development of these and other techniques for the preparation of stable genetically transformed monocots such as maize could potentially revolutionize approaches to monocot breeding.

S~W OF THE IWENTION The present invention addresses one or more of the foregoing or other shortcomings in the prior art 0 providing methods and compositions for the preparation of stably transformed, monocotyledonous cells and subsequent regeneration of fertile, transgenic plants and progeny, particularly maize.

It is therefore a particular object of the present invention to provide techniques that will allow one to prepare Wansg;nV, fertile monocots such as maize which are preferrably diploid and which have been stably transformed "ough the introduction of a desired gene into its genome.

Me present invention thus relates generally to methods for Me p roduction of transgenic plants. As used herein, Me term transgenic plants is intended to refer to plants that have incorporated exogenous genes or DNA sequences, including but not limited to genes or DNA sequences which are perhaps not normally present, genes not normally transcribed and translated ("expressed") in a given,cell~type. or an other genes of DNA sequences Mich one desKes to introduce into Me non-transformed plant, such as Wes which may normally be present in the non-transformed plant but which one desires to have altered expression.

Memplary genes which may be introduced include, for examplOONA sequences or genes from another species, or even genes or sequences which originate with or are present in Me same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques.

However, Me term exogenous, is also intended to refer to genes which we not normally present in the cell being transformed, or perhapsn-simply not present in the form, structure, etc., ad found in the transforming DNA segment or gene, or~qenes which are normally present yet which one desires,'-O;g., to ham overexpressed. Thus, the term "exogenoush gene or DNA is intended to refer to any gene or DNA segment Mat is introduced into a recipient cell, r egardless, of Kether a similar gene may already be present in such a cell.

An initial step in Me production of fertile transgenic plants is We obtaining of a DNA composition, e.g., vectors, plasmids, linear DM fragments, and the like, a component of which is to be delivered to recipient monocotyledonous cells. DM segments for use in transforming such cells will, of course, generally comprise the gene or genes which one desires to introduce into the cells. These genes can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired.

The construction of vectors which may be employed in practicing the present invention is generally within the skill of the art. (See generally, refs 79, 80).

Preferred constructs will generally include a plant promoter such as the CaW 35S promoter (68), or others such as CaW 19S (69), nos (70), Adh (71), sucrose synthase (72), those associated with the R gene complex (55), or even tissue specific promoters such as root cell promoters (73) and tissue specific enhancers (74).

Constructs will also include the gene of interest along with a 31 end such as that from Tr7 or nos (75), or the like. Regulatory elements such as Adh intron 1 (76), sucrose synthase intron (77) or " omega element

(78), may further be included "ere desired.

Certain elements may find utility when incorporated into genomes, even without an associated expressible gene. For example, transposons such as Ac, Ds or Mu are elements which can insert themselves into genes and cause unstable mutations. This instability apparently results from subsequent excision of the element from the mutant locus during plant or seed development. For a review covering the use of transposon elements, see references 56 and 57. These elements, particularly Ac, may be introduced in order to inactivate (or activate) and thereby "tag" a particular trait. once tagged, the gene with this trait may be cloned, e.g., using the transposon sequence as a PCR primer together with PCR gene cloning techniques (58,59). Once identified, Me entire gene(s) for Me particular trait, including control or regulatory regiom whare desired, may be isolated, cloned and manipulated as desired prior to re-introduction.

Me generation and use of recipient cells is believed to be an important aspect of We invention. As used herein, Me term "recipient cell" is intended to refer to monocot cells that are receptive to transformatin ~~and subsequent regeneration into stably transformed, fertile monocot~~ plants. ~~The inventors thus~~ propose Mat not all cells present in a population of cells subjected to transforming events will be "recipient" to successful transformation and regeneration. However, it is proposed that through the application of Me techniques disclosed herein, one will be enabled-to obtain populations which contain sufficient numbers of recipient dells to allow for successful stable transfo rma tion and regeneration.

Certai* techniques are disclosed which may enrich for recipient cells. For example, it is believed that Type II callus development, followed by manual selection and culture-of friable, embryogenic tissue, results in an enrichment"91 recipient cells. Suspension culturing, particularly using Me media disclosed in Table I herein, may also improve Me ratio of recipient to non-recipient cells in a" given population.

Me frequency of occurrence of cells receiving DNA W is believed1to be low. Moreover, it is most likely that not all rq lls receiving DNA segments will Wc±pient ce result in a transformed cell wherein We DNA is stably integrated into the plant genome and/or expressed. Some may show only initial and transient gene expression.

However, it7is proposed that certain cells from virtually any monocot species may be stably transformed through the W 91/02071 PCIUUS90/OM62 application of the techniques disclosed herein.

The most preferred monocot will be the cereals such as maize. With respect to maize, the inventors propose that many of the techniques of the invention will be applicable to maize varieties in general, whether inbred, elite inbred or hybrid varieties. It should be pointed out, though, that not all cell lines developed out of a particular variety or cross will necessarily show the same degree of stable transformability. For example, the present invention is exemplified through the use of A188 x B73 cell lines developed by standard techniques out of an A188 x B73 cross. The lines identified as SC716 and SC82 are examples of cells lines which were developed from an A188 x B73 cross as described hereinbelow.

However, a num ber of other cell lines developed from the same cross have not as yet proven to be stably transformable. Thus, stable transformability may not be immediately apparent with some lines even from the same cross. (2 out of about 12 A188 x B73 lines have proved to be stably transformable and yield fertile transgenic plants; about 16% of the lines). Thus, "ere one desires to prepare transformants to a particular cross or variety, it will generally be desireable to develop several cell lines from the particular cross or variety (e.g., 8 to 10), and subject all of the lines so developed to the transformation protocols hereof.

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. Marker genes code for

phenotypes that allow cells which express the marker gene to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on "ether the marker confers a trait which one can select for by chemical WO 91102071 PCF/US90104462

means, Ke.,-~throuqh the use of a selective agent (e.g., an herbicide, antibiotic, or Me like), or "ether it is simply-a trait Mat one can identify through observation or testing (e.g., Me R-locus trait). Of course, many ex amp les of suitable marker genes are kn own to the art and can be empl" in- Me practice of the invention.

PossibIA selectable markers for use in connection with Me present ' invention include but are not limited to a neo gone (82) %ich codes for kanamycin resistance and can beltelected for using kanamycin, G418, etc.; a bar Ono which codes for bialaphos resistance; a mutant EPSP Qne (67) which encodes glyphosate resistance; a nitrila se gene %ich confers resistance to bromoxynil (83); a mut" a6etolactate synthase gene (MS) which confers imidazolinone or sulphonylurea resistance (60); or a methotrexate resistant DHFR gene (61). Were a mutant WSP synthase gene is employed, additional benefit-may-be"-realized-through-We incorporation of a suitable chloroplast,trahsit peptide (CTP; see ref. 62).

Ex emp lary screenable markers include a P- glucuronidase or AM gene (GUS) which encodes an enzyme for which "ious chromogenic substrates are known or an R-locus gens; Which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (59)," included within the temso"selectable or screenable marker,genes" ne also genes which encode a secretable : m marker whoss`secrkion can be detected as a means of identifying or selecting for transformed cells. Examples include markers Wich =code a secretable antigen that can M ide=jfied.by antibody interaction, or even secretable =Z"es Thich can be detected catalytically.

Secretable, proteins fall into a number of classes, including M11, diffusible proteins detectable, e.g., by i~'- ELISA, small active enzymes detectable in extracellular solution, or proteins which are inserted or trapped in the cell wall.

Of course, in light of this disclosure, numerous other possible selectable and/or screenable marker genes will be apparent to those of skill in the art.

Therefore, the foregoing discussion is intended to be exemplary rather than exhaustive. Although the present disclosure is exemplified in detail through the use of the ban and/or GUS genes, the applicable techniques for making and using any other screenable or selectable marker gene will be within the skill in the art in light of the present disclosure.

An illustrative embodiment of marker genes capable of being used in systems to select transformants is the bar gene from Streptomyces, such as from the hMoscoacus species. The bar gene codes for phosphinothricin acetyl transferase (") that inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (29, 47) causing rapid accumulation of ammonia and cell death. Success in use of this selective system in the case of monocots was unexpected because of the major difficulties which have been encountered in transformation of cereals (36).

Where one desires to employ a bialaphos resistance gene in the practice of the invention, the inventors have discovered that a particularly useful gene for this purpose is the bar gene obtainable from species of Streptomyces (MCC No. 21,705). Me cloning of the bar gene has been described (29, 45) as has the use of the bar gene in the context of plants other than monocots (10, 11). However, in light of the techniques disclosed herein and the general recombinant techniques which are known in We a", the introduction and use of an of the foregoing or other genes is now possible.

Me use of a gene from the maize R gene complex is proposed as a particularly useful screenable marker.

The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one or as many as.four R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. The present inventors have applied a gene from Me R gene qpmplex to maize transformation because it is viable, it ip a naturally occurring product in maize, and it is visualized without Me need for additional assays.

Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line is dominantsfor We enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, Al, A2, Bz1 and Bz2), but recessive at the R locus, any cell from that line can be emp loyed as a recipient for transformation.

Ex emp lary lines include rg-Stadler in Wisconsin 22 and TR112, a K55 derivative which is r-g, b, Pl.

Me inventqrs further propose that R gene regulatory regions may be emp loyed in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at We R locus Man at any other locus (63). It is contemplated Mat regulatory regions obtained from regions 51 to We structural R gene could be very valuable in directing We expression of genes for, e.g., insect resistance, herbicide tolerance or other protein coding regions. For the purposes of Me present invention, it is believed "at a, of We various R gene family members may be successfully employed (e.g-, P, S, Lc, etc.). However, Me most preferred will generally be Sn (particularly SnOo13). Sn is a dominant m emb er of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of anthocyanin pigments in certain seedling and plant cells. Thus, its phenotype is similar to R.

1 The choice of the particular DM segments to be delivered to the recipient cells will often depend on the purpose of the transformation. one of the major purposes of transformation of crop plants is to add some commercially desirable, agronomically important traits to the plant. Such traits include, but are not limited to, herbicide resistance, increased yields, insect and disease resistance, physical appearance, food content and makeup, etc. For example, one may desire to incorporate one or more genes encoding herbicide resistance. The bar and glyphosate tolerant EPSP synthase genes are good examples. A potential insect resistance gene which can be introduced includes the Bacillus thuangiensis crystal toxin gene (86), which may provide resistance to pests such as lepidopteran or coleopteran.

Genes encoding proteins characterized as having potential insecticidal activity, such as the cowpea trypsin inhibitor (CpTI; 88) may find use as a rootworm.

deterrent; genes encoding avermectin (84,85) may prove particularly useful as a corn rootworm deterent.

Furthermore, genes encoding lectins may confer insecticide properties (e.g., barley, Meat germ agglutinin, rice lectins, see ref. 81), Mile others may confer antifungal properties (e.g., hevein, chitinase, 1 see. e.g., ref. 65). It is proposed that benefits may be realized in terms of increased resistance to mold temperatures through the introduction of an "antifreeze" protein such as that of the Winder Flounder (87).

Ultimately, Me most desirable "traits" for intro- duction into,a monocot genome may.be homologous genes or gene families whieh encode a desired trait (e.g., increased yield per acre) and which are introduced under Me control of novel promoters or enhancers, etc., or perhaps"even homologous or tissue specific (e.g., root specific) promoters or control elements.

Me invention thus contemplates that particular benefits may be realized by the transformation of plant cells with any expressible gene, and is not intended to be limited to the use of marker genes. As used herein, an "expressible gene" is any gene that is capable of being translated into a protein, expressed as a trait of interest, or We like, etc., and is not limited to selectable, screenable or non-selectable marker

genes.

Me invention also contemplates Mat, "ere both an expressible jene that is not necessarily a marker gene is employed in combination with a marker gene, one may employ the separate genes on either the same or different DNA segments for transformation. In the latter case, Me different vectors are delivered concurrently to recipient cells to maximize cotransformation.

In certain embodiments, recipient cells are selected following growth, in culture. Were employed, cultured cells will P referably be grown either on solid supports or in the fWof liquid suspensions. In either instance, nutrients my be provided to the cells in the form of media, and environmental conditions controlled.

There are-many types of tissue culture media comprising amino acids, salts, sugars, hormones and vitamins. Most of the,media employed in Me practice of the invention will haVe s*e similar components (see, e.g., Table 1 herein belM , We media differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide.

Various types of media suitable for culture of plant cells have been previously described. Ex amp les of these media include, but are not limited to the N6 medium io described by Chu, et al. (5) and the MS media (30). In an exemplary embodiment for preparation of recipient cells, the inventors have modified these media (see, Table 1). A preferred hormone for such purposes is dicamba or 2,4-D. However, other hormones may be employed, including NAA or NAA + 2,4-D. Modifications of these and other basic media may facilitate growth of recipient cells at specific developmental stages.

An exemplary embodiment for culturing recipient corn cells in suspension cultures includes using embryogenic cells in Type II callus, selecting for small (10-30 p) isodiametric, cytoplasmically dense cells, growing the cells in suspension cultures with hormone containing media, subculturing into a progression of media to facilitate development of shoots and roots, and finally, hardening the plant and readying it metabolically for growth in soil. For use in transformation, suspension culture cells may be cryopreserved and stored for periods of time, thawed, then used as recipient cells for transformation.

An illustrative embodiment of cryopreservation methods comprises the steps of slowly adding cryoprotectants to suspension cultures to give a final concentration of 10% dimethyl sulfoxide, 10% polyethylene glycol (6000M), 0.23 M proline, and 0.23 M glucose. The mixture is then cooled to -350C at 0.50C per minute.

After an isothermal period of 45 minutes, samples are placed in liquid N.. (Modification of methods of Withers and King (49); and Finkle, et al.jQ). To reinitiate suspension cultures from cryopreserved material, cells may be Maweg rapidly and pipetted onto feeder plates similar to those described P Modes, et al. (38).

one ~d%ent of cultured plant cells that can serve as recipient cells for transforming with desired DNA segments, such as Mose which comprise expressible genes, includes corn cells, more specifically, cells from Zea ma, L. Somatic cells are of various types.

Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which will typically not respond in such a fashion. An example of non-embryogwic cells are certain Black Mexican Sweet (BMS) coin cells, and Mese have been successfully transformed by microprojectile bombardment using the neo gene followed P selection with the aminoglycoside, kanamycih (22). However, %is BMS culture was not found to be regenerable, and general use of kanamycin may be hMered%Q endogenous resistance of maize (19).

other recipient cell targets include, but are not limited to4 meristem cells, Me I and II calli and gametic cells such as microspores and pollen. Pollen, as well as its pre cut sor cells, micropsores, may be capable of functioning as recipient cells for genetic transformation, or as vectors to carry foreign DNA for incorporation dur ing fertilization. Direct pollen transformation would obviate Me need for cell culture.

Meristematic cells (i.e., plant cells capable of continual ~ W11 division and characterized a an undifferentiated cytological appearance, normally found at growing points or tissues in plants such as root tips, stem apices, lateral buds, etc.) may represent another type of recipient plant cell. Because of their undiffer- entiated growth and capacity for organ differentiation and totipotency, a single transformed meristematic cell could be recovered as a whole transformed plant. In fact, it is proposed that embryogenic suspension cultures may be an in vitm meristematic cell system, retaining an ability for continued cell division in an undifferentiated state, controlled by the media environment.

The development of embryogenic maize calli and suspension cultures useful in the context of the present invention, e.g., as recipient cells for transformation, has been described in U.S. Serial No. 06/877,033, filed 06/07/86, incorporated herein by reference.

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as by A"acterium infection or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles, etc. Acceleration methods are generally preferred and include, for example, microprojectile bombardment and the like.

Electroporation has been used to transform corn protoplasts (17).

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

A particular advantage of microprojectile At WO 91102071 PCFIUS90104462 18 bombardment, in addition to it being an effective means of reproducibly-stably transforming monocots, is that neither Me isMation of protoplasts (8) nor the susceptibility of A"acteaum infection is required.

An illustrative emWdiment of a method for delivering DNA into maize cells 0 acceleration is a Biolistics Particle Delivery System, whilch can be used to propel particles coated with D~ through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with corn cells cultured in suspension.

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing Me cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between We gun and the cells to be bombarded.

Through the u se of techniques set forth herein one may obtain up to 100 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. Me number of cells in a focus which express the exogenow gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

After effecting delivery of exogenous DNA to recipient cells by any of We methods discussed above, a preferred sW is to identify the transformed cells for further cultdring and plant regeneration. This step may include assaying cultures directly for a screenable trait or 0 exposing We bombarded cultures to a selective agent or agents.

An example of a screenable marker trait is the red pigment produced under Me control of the R-locus in maize.- This pigment may be detected 0 culturing cells on a solid support containing nutrient media capable of supporting growtV" this stage, incubating the cells at e.g., 180 C. and greater than 180 gE C2 sec-1, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g., for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/1 bialaphos. Mile ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the.exposure to the selective agent may be cultured in media that supports regeneration of plants. An example of suitable media is a modification of MS media (Table 1). Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

Mgeneration typically requires a progression of media lose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to Me more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 Pp" COV and 250 microeinsteins M-2. S-1 of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue cul tur e vessels. An illustrative embo diment of such a vessel is a petri dish.

Regenerating plants are preferably grown at about 19 to 280C. After the regenerating plants haw reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing- To confirm We presence in the regenerating plants of traits delivered to Me recipient cells through the application of exogenous DNA, alone or in conjunction with marker genes, assays for expression of said genes may be perfo rme d, e.g., by testing parts of the regenerated plants. Exemplary parts which may be assayed are leaves. A typical transformant assay includes contacting regenerating plants or extracts of plants with a substrate Mat is acted upon by the transforming gene product. At "is stage of development, the plants will not be lethally affected by such an assay. Removal of small portions of Me plants does not cause their death or iQerf me with further development.

In one study, , plants were regenerated from trans- formants of an A188 x B73 suspension culture line (SC82) transformants, and these plants exhibited a phenotype expected of Me genotype of hybrid A188 X B73 from which Me callus and culture were derived. Me plants were similar in height to seed-derived A188 plants (3-5 ft tall) but had B73 traits such as anthocyanin accumulation in stalks and prop roots, and the presence of upright leaves. It would also be expected that some traits in the transformed plants would differ from their source, and indeed some variation will likely occur.

0 In an exemplary embodiment, the proportion of regenerating plants derived from transformed callus that successfully grew and reached maturity after transfer to the greenhouse was 97% (73 of 76). In one example, at least 50 viable progeny were recovered from , plants. RO plants in the greenhouse were tested for fertility by backcrossing the transformed plants with seed-derived plants by pollinating the P

D ears with pollen from seed derived B73 plants and this resulted in kernel development. Note, however, that kernels on transformed plants may require embryo rescue due to cessation of kernel development and premature senescence of plants.

To rescue developing embryos, they are excised from surface-disinfected kernels 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In an illustrative embodiment of embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that were cultured for one week on media containing the above ingredients along with 10_5M abscisic acid and then transferred to hormone-free medium for germination.

Progeny may be recovered from the transformed plants and tested for expression of the exogenous expressible gene by localized application of an appropriate substrate to plant parts such as leaves. In the case of bar transformed plants, it was found that-transformed parental plants Q) and their progeny (Rj) exhibited no bialaphos-related necrosis after localized application of Me herbicide Basta to leaves, if there was functional PAT activin,in the;plants as assessed by an in vitro enzymatic assay. In one study, of 28 progeny (Rj) plants testedV 50%'jN=14) had PAT activity. All PAT positive progeny tested contained bm, confirming that the presence ofilhe enzyme and Me resistance to bialaphos were associated with Me transmission through the germline of the marker gene. The nonchimeric nature of We callus and Me parental transf ormants (Q was suggested germline transmission and the identical Southern bl" hybridization patterns and intensities of Me trans"ming DNA in callus, , plants and R, progeny Mat segregated for Me transformed gene.

Genomic DNA may be isolated from callus cell lines and plants to determine Me presence of the exogenous gene through the use of techniques well known to those skilled in Me a". Note, that intact sequences will not always be present, presumably We to rearrangement or deletion of.,sequences in the cell.

We invenOrs have been successful in producing fertile trahsgenic monocot plants (maize) "ere others have faileK' Aspects of Me methods of the present inventioh, for producing We fertile, transgenic corn plants comprise, but are not limited to, development of suspension cultures of recipient cells using media conducive talspecific growth patterns, choice of selectIve systems Mat permit efficient detection of transformation; modifications of acceleration methods to introduce genetic4ectors with exogenous DNA into cells; invention otmethods to regenerate plants from transformed cells at a hip frequency; and the production of fertile transgenic-plants capable of surviving and reprodupin:

" 91/02071 PCr/US90/OU62 DEFINITIONS Callus - Proliferating mass of cells or tissue Y Atro.

Type I - A compact, slow growing, heteromorphic callus (embryogenic/organogenic) which retains meristematic activity in regions of organized tissue.

Type II - A friable, fast growing embryogenic callus composed of aggregates of small isodiametric cells with dense cytoplasm. often contains small embryoids attached to the underlying callus by a suspensor.

Embryogenic Callus - A type of callus capable of differentiating into somatic embryos.

Germinal Cells (Gametes). - Cells of an organism which are capable of transferring their genetic information to the next generation.

Genotypg - The genetic c M lement of an organism.

Heterologous D= - DW from a source different than that of the recipient cell.

Homologous D - DM from the same source as that of the recipient cell.

Hybrid - Progeny resulting from a cross between parental lines.

Inbred Lines - Organisms that are genetically homogeneous (homozygous) resulting from many generations of self crossing.

In Vitro In the laboratory.

In Vivo In the living organism.

~o cot Plants having a single cotyledon (the first leaf of the embryo of seed plants); examples include cereals such as maize, rice, wheat, oats and barley.

Non-Embryogenic Callus - A type of callus composed of undifferentiated, often highly vacuolated cells which are unable to be induced to form embryos.

Phenotype - Traits exhibited by an organism resulting from the interaction of genotype and environment.

Protoplast - Plant cells exclusive of the cell walls.

Somatic Cells - Body cells of an organism, exclusive of ~,Ir' ~ WO 91102071 PCr/US90104462 24 germinal cells.

Transformation - Acquisition of new genetic coding sequences Me incorporation of added (exogenous) DM.

Transaenic organisms (plants or animals) into which new DM sequencea are integrated.

BRI" D~SCRIPTION OF THE DRAWINGS PIG. 1. Schematic renresentation of Wasmids (vectors) used in bombardment e=eriments. The plasmids 0 have been named (J pDPG161 which contains bar, and (B) pDPG208 which contains uW (the gene which encodes P- glucurMidage (WS)). Letters designate Me locations of various restriction sites, locations which may be cleaved by restriction endonucleases, E, EcoRI; H, HindIII; B, BanHI; S, SmaC A more detailed map of pDPG165 is shown in (C), of JPGibS in (D). In (E) is shown a restriction map of pAGUS1, also known as pDPG141, in which a the 51- noncoding and 51-coding sequences were modified to incorporate Me Kozak consensus sequence and a HindIII restriction site! In (F) is shown a restriction map of pDPG237, a plasmid which contains SnOo13 cD%, and in (G) is shown_a map of pDPG232, a plasmid which incorporates Rsn cD= along with a 35S promoter and a Tr7 31 end.

FIG. 2. ~earance of cell colonies which emercre on selection plates with bialaphos. Such colonies appear 6-7 weeks after bombardment. (A) SCS2 bialaphos- resistant colon selected on 1 ng/1 bialaphos. (B) EmbryogeCOSC82 bialaphos-resistant callus selected and maintained on 1 mg/1 bialaphos.

FIG. 3. Phosphinothricin acetyl transferase (PAT1 activitv in embryogenic SC82 callus transformantS desinated El-Ell and a nonselected control (E . 25 gg of protein extract were loaded per lane. B13 is a BMS- bar transformant. BMS is Black Mexican Sweet corn.

Activities of the different transformants varied approximately 10 fold based on the intensities of the bands.

FIG. 4. Intearation of the bar gene in bialaphos= resistant SC82 callus isolates El-Ell. DNA gel blot of

genomic DNA (4 gg/digest) from El-Ell and a nonselected control (EO) digested with EcoRI and HindIII. The molecular weights in kb are show on the left and right.

The blot was hybridized with 32p_labeled bar from pDPG165 (-25xlO6 Cerenkov cpm). Lanes designated I and 5 copies refer to the diploid genome and contain 1.9 and 9.5 pg respectively of the 1.9 kb bar expression unit released from pDPG165 with EcoRI and HindIII.

FIG. 5. PAT Activitv in Protein Extracts of R Plants. Extracts from one plant derived from each of the four transformed regenerable callus lines from a suspension culture of A188 x B73, SC82 (ElO, Ell, E2/E5j and-E3/E4/E6) were tested for PAT activity (The designations E2/E5 and E3/E4/E6 represent transformed cell lines with identical DNA gel blot hybridization patterns; the isolates were most likely separated during the culturing and selection process.) Protein extracts from a nontransformed B73 plant and a Black Mexican Sweet (BMS) cell culture bar transformant were included as controls. Approximately 50 micrograms of total protein was used per reaction.

-1FIG. 6. DNA Gel Blot Analysis of Genomic DNA from Transformed Callus and Correspondinc R. Plants Probed with bar. Genomic DNA was digested with EcoRI and HindIII, which released the 1.9 kb bar expression unit (CaW 35S promoter-bar-Tr7 31-end) from pDPG165, the plasmid used for microprojectile bombardment transformation of SC82 cells, and hybridized to bw. The molecular weights in M are shown on the left and right.

Lanes designated E3/E4/E6, Ell, E2/E5, and ElO contained gg of either callus (C) or N plant DM. The control lane contained DM from a nontransformed A188 X B73 plant. The lane designated Hl copy" contained 2.3 pg of Me 1.9 M EqKRI/HindIII fragment from pDPG165 representing one copy per diploid genome.

Y' FIG. 7. PAT Activitv and D= Gel Blot Analvsis of Segregating -Progeny of E2/E5 R, Plants. (A) Analysis of activity in ten progeny (Anes a-j) and a nontransformAd control plant (lane Q. Lanes designated a, b-h, i, and j contained protein extracts from progeny of separate parental % plants. The lane designated callus contained protein extract from E2/E5 callus.

Approximately 25 micrograms of total protein were used per reaction. (B) DM gel blot analysis of genomic DM isolated from We ten progeny analyzed in A. Genomic DM (5 gg/lane) was digested with SmaI, which releases a 0.6 kb fragment containing bar from pDPG165, and hybridized with bw probe. The lane designated 0 contained DM from the Q parent of progeny a. The lane designated I copy contained pDPG165 digested with SmaI to represent approximately 1 copy of Me 0. 6 " fragment per diploid genome (0. 8- pg) FIG. 8. Histochemical determination of GUS activity in bar-transformed SC82 callus line Y13. This bialaphos-resistant callus line, Y13, which contained intact GUS coding sequences was tested for GUS activity three months post-bombardment. In this figure, differential staining of Me callus was observed.

FIG. 9. Intenation of exogenous genes in bialapho.s-resistint SC716 isolates Ri-R21. (A) DM gel blot of genopicaDM (6 gg/digest) from transformants isolated from suspension culture of A188 x B73 (SC716), designated Rl-R21, were digested with EcoRI and HindIII and hybridized to 32 P-labeled bar probe (_IOX,06 Cerenkov cpm). Molecular weight markers in kb are show on the left and right. Two copies of the bar expression unit per diploid genome is 5.7 pg of the 1.9 kb EcoRI/Hind fragment from pDPG165. (B) The blot from A was washed and hybridized with 32 P-labelled GUS probe (-35xlO 6 Cerenkov cn). Two copies of the 2.1 kb GUS-containing EcoRI/HindIII fragment from pDPG208 is 6.3 pg.

FIG. 10. Functional Expression of Introduced Genes in Transformed RO and _Plants. (A) Basta R resistance in transformed & plants. A'Basta R solution was applied to a large area (about 4 x 8 cm) in the center of leaves of nontransformed A188 x B73 plant (left) and a transgenic A E3/E4/E6 plant (right). (B) BastaR resistance in transformed R, plants. BastaR was also applied to leaves of four R, plants; two

plants without bar (left) and two plants containing bar (right). The herbicide was applied to R, plants in 1 cm circles to four locations on each leaf, two on each side of the midrib. Photographs were taken six days after application. (C) GUS activity in leaf tissue of a transgenic , plant. Histochemical determination of GUS activity in leaf tissue of a plant regenerated from cotransformed callus line Y13 (right) and a nontransformed tissue culture derived plant (left).

Bar = 1 cm. (D) Light micrograph of the leaf segment from a Y13 plant show in Q, observed in surface view under bright field optics. GUS activity was observed in many cell types throughout the leaf tissue (magnification = 230X). (E) Light micrograph as in (D) of control leaf FIG. 11. Mature RO Plant. Developing Kernels an - Proqenv.. (A) Mature transgenic S plant regenerated from an E2/E5 callus. (B) Progeny derived from an E2/E5 plant Q embryo rescue; segregant bearing the resistance gene on Me right, and lacking the gene on the left. (C) Using pollen from transformed R, plants to pollinate B73 ears, large numbers of seed have been recovered. (D) A transformed ear from an R, plant crossed with pollen from a non-transf ormed inbrePplant.

DESMIPTION OF THE PREFEWED EMBODIMENT9 For the.first time, fertile transgenic maize plants have been produced, opening Me door to new vistas of crop improvement based on in Otro genetic transformation. The inventors have succeeded "ere others failed by combining and modifying numerous steps in Me overall-process leading from somatic cell to transgenjc plant. Although the methods disclosed herein are part of a unified process, for illustrative purposes they my be yubdivided into: culturing cells to be recipients for exogenous DNA; cryopreserving recipient cells; constructing vectors to deliver the DNA to cells; delivering M to cells; assaying for successful transformations; using selective agents if necessary to isolate stable transformants; regenerating plants from transformants; assaying those plants for gene expression and for identification of Me exogenous DNA sequences; determining whether We transgenic plants are fertile; and producing offspring of Me transgenic plants. The invention also relates to transformed maize cells, transgenic plants and pollen produced by said plants.

A. Tissul-Culthre Tissue culture requires media and controlled environments. "Media" refers to the numerous nutrient mixtures that' are used to grow cells in O"o, that is, outside of Me intact living organism. The medium is usually a suspension of various categories of ingredients (salts, amino acids, hormones, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth will also vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bactoagar and Gelgro are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, maize cells will grow in suspension, but regeneration of plants requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH, but also by "ether media is solid or liquid. Table 1 illustrates the composition of various media useful for creation of recipient cells and for plant regeneration.

B. Culturing Cells in Suspension to be Recipients for Transformation

It is believed by the inventors that the ability to prepare and cryopreserve suspension cultures of maize cells is an important aspect of the present invention, in that it provides a means for reproducibly and successfully preparing cells for transformation. The studies described below set forth techniques which have been successfully applied by the inventors to generate transformable and renegerable suspension

cultures of maize cells.. A variety of different types of media have been developed P Me inventors and employed in carrying out various aspects of the invention, including in particular, Me development of suspension cultures. The following table, Table 1, sets forth the composition of Me media pref erred P Me inventorssfor carrying out these aspects of the invention.

Table 1: Illustrative Embodiments of Tissue Culture Xedia Which are Used for Type II Callus Development, Development of Suspension Cultures and Regeneration of Plant Cells (Specifically Xaize Cells) Medium id. Optimal Other Number MS* N6 Sucrose pH Components** 52 + 2% 6.0 0.25 mg thiamine 1 %2,4-D 10 ABA Bactoagar 101 + 3% 6.0 100 mg Wo-inositol v Bactoagar

142 + 6% 6.0 5 mg BAP v 0. 186 =g NAA 0. 175 mg I= 0.403 mg 2-IP 200 mg Wo-inositol Bactoagar 163 + 3% 6.0 3.3 mg dicamba v 100 mg Mo-inositol Bactoagar 171 + 3% 6.0 0.25 mg 2,4-D v 10 mg BAP 100 mg Wo-inositol Bactoagar 173 + 6% 6.0 5 mg BAP v 0. 186 mg NAA 0. 175 mg 1AA 0.403 mg 2-IP 10-5 M AM 200 mg Wo-inositol Bactoagar

177 + 3% 6.0 0.25 mg 2,4-D v 10pg BAP 5M ABA 100 mg Wo-inositol Bactoagar 2 01 + 2% 5.8 25 = proline v 1 mg 2,4-D 100 mg casein 55 GelgroR hydrolysate 205 + 2% 5.8 25 AM proline v 0.5 mg 2,4-D 100 mg casein Gelgro R hydrolysate 227 + 2% 5.8 25 AM proline v 13.2 mg dicamba 100 mg casein GelgroR hydrolysate 401 3% 6.0 0.25 mg thiamine 1 mg 2,4-D 2 mg =

200 mg casein hydrolysate 500 mg K sulfate 100 mg myo-inositol 400 mg K phosphate (monobasic) 402 + 3% 6.0 0.25 mg thiamine 25 AM proline 1 mg 2,4-D 200 M casein hydrolysate 500 mg K sulfate 400 mg K phosphate (monobasic) 100 mg Wo-inositol 35 409 + - 3% 6.0 0.25 mg thiamine

- 25 = proline mg dicamba 200 mg casein hydrolysate

.500 mg K sulfate 400 mg K phosphate (monobasic) 100 mg Wo-inositol 501 2% 5.7 Clark's GelgroR Basic MS medium described in reference 30. The 50 medium described in ref. 30 is typically modified P A decreasing. Me M.NO 3 fro 1.64 g/1 to 1.55 g/1, and omitting Me pyridoxine RC1, nicotinic acid, Mo- inositol and glycine.

55 + = present; absent; ~tamins NAA = Napthol Acetic Acid IM = Indole Acetic Acid 2-IP 2, isopentyl adenine 2,4-D 2, 4-Dichlorophenoxyacetic Acid BAP = 6-benzyl aminopurine ABA = abscisic acid Basic medi= described in reference 6 EX M le 1: Initiation of the Suspension Culture GII(A188=73)716 (designated SC716) for Use A Transf ormation This ~ple describes the development of a maize suspension culture, designated SC716, which was employed in various of Me transformation studies described hereinbelow. The Me II tissue used to initiate the cell suspensimwas derived from immature embryos of A188 x B73 plated onto N6-based medium with I mg/ml 2,4-D (201; se&Table 1). A Me II callus was initiated by visual selection of fast growing, friable embryogenic cells. The suspension was initiated within 6 months after callus initiation. Tissue chosen from the callus to initiate Me suspension consisted of very undifferentiated M e II callus, the characteristics of this undifferentiated tissue are the earliest stages of embryo development along with the soft, friable, undifferentiated tissue underlying it.

. Approximately one gram of tissue was added to 20 mls of liquid medium. In this example, the liquid medium was medium 402 to T which different slow-release hormone capsule tre" nts were added (see Example 12 below).

These capsul treatments included 2,4-D, ", 2,4-D plus " , and 2 " capsules. One flask was initiated for each of We different 402 media plus hormone combinations Men 7 days each culture was subcultured into fresh medium by transferring a small portion of the cellular suspension to a new flask. This involved swirling Me original flask to suspend the cells (which tend to settle to Me bottom of the culture vessel), tilting Me flask on its side and allowing the denser cells and cell aggregates to settle slightly. One ml of packed cells was Men drawn off from this pool of settled cells together with 4 mls of

conditioned medium. A sterile ten ml, wide tip, pipet was used for this transfer (Falcon 7304). Any very large aggregates of cells which would not pass easily through the pipet tip were excluded. If a hormone capsule was present, it was also transferred to the new flask.

After approximately 7 weeks, the loose embryogenic cell aggregates began to predominate and fragment in each of the cultures, reaching a state referred to as "dispersed." The treatment which yielded the highest proportion of embryogenic clusters was the 402 medium plus a NAA capsule. After the cultures became dispersed and were growing at a fast rate, doubling approximately every two to three days as determined by increase in packed cell volume, a one ml packed cell inoculum. from each culture was transferred into 401 medium using a ten ml narrow tip pipet (Falcon 7551). These transfers were performed about every 3; days. An inoculum from the 402 plus 2,4-D plus NAA capsules culture was also used to initiate a culture in 409 medium (402 minus 2,4-D and plus 10mg/l dicamba) either with or without 1 ml coconut water (Gibco 670-8130AG).

The most dispersed cultures were cryopreserved after 2 weeks, 2 months or 5 months.

The culture grown on 409 with coconut water was brought out of cryopreservation eight months later and thawed, cultured for two weeks on solid 201 culture medium using BMS as a feeder layer (38) and transferred to media 409 without coconut water. Me culture was maintained by subculturing twice weekly, using 409 media, by the method described above.

Example 2: Initiation of the Suspension Culture (A188 X B73)82 (designated SC82) for Use in Transformation This Example describes the development of another cell line employed in various of We transformation studies set for" below, termed SC82. In the development of SC82, inoculum for suspension culture initiation was visually selected from a Type II callus that was derived from imma" e embryos plated on a N6-based medium containing 13.2 mg/l dicamba (227) (Table 1) . We suspension culture was initiated within 3 months of initiation a Me Me II callus. Small amounts (50-100 mg) of callus dAtinguishable 0 visual inspection because of Us highly proembryonic morphology, were isolated from more mature or organized structures and inoculated "o-a 50 ml.flask containing 5 mls of filter-sterilized conditioned medium from the various GII J188 x B73) 716 suspension cultures (402 medium with four types of capsule treatments and 409 medium).

After one week, this 5 ml culture was sieved through a 710 mi"n mesh and used to inoculate 20 mls of corresponding fresh and filter-sterilized conditioned medium from Me established GII J188 x B73) 716 cultures in 150 ml flasks. After one week or more of growth, two mls of packed cells were subcultured to fresh media by Me method described above. The suspension culture maintained on 409 a this method was then cryopreserved within 3 months. The original cell line, which was maintained On 409 (not a reinoculated cryopreserved culture) was' used in experiments I and 2 months later which resulted in stable transformation and selection (see Table 2 below). Me cryopreserved culture was used f or experiment 6 (see Table 2 below) .

i.

C. Slow Release Plant Hormone Capsules Studies following the fate of radioactively labelled plant hormones (2jOD and ") showed that within two days corn Alls absorb most of Me auxins present in suspension culture media. This problem of hormone ';c depletion can be overcome by spiking the cultures with a small amount of auxin every other day. However, spiking cultures is very time consuming Men done on a large scale and also increases the risk of contamination as the culture vessels must be opened frequently. Slow release plant hormone capsules were developed to overcome these problems. In summary, these capsules comprise a plant hormone, usually in a crystalline state, encapsulated in a silicone matrix surrounded by a silicone limiting membrane. The rate of hormone release is controlled by the size of the diffusible area and the thickness of the membrane. They have the advantages of 1) supplying hormones at an acceptable and predictable rate (e.g.,

- 100 gg/20 ml culture media/day, 2) they are of a convenient size (e.g., 0.5 - 1.5 cm in length) for use in liquid or solid culture medium, 3) they are very durable and easily sterilized by autoclaving, and 4) they can be stored dry until needed.

The present formulation involves the controlled release of a plant hormone or selective agent for a plant tissue culture from an inner matrix containing crystals of the desired agent through an outer diffusion limiting membrane. A preferred embodiment of the formulation is to mix 30% dry crystals of the desired agent with 70% (w/w) room temperature vulcanizing (RTV) silicone which is then injected into silicone tubing having an appropriate diameter and wall thickness for the desired release rate of the desired agent. (The preferred agents for employing in connection with the slow release capsules are 2,4-D and " , and the preferred dimensions are 0.062" ID x 0.125" OD).

The WV silicone is then polymerized at room temperature-or at a higher temperature to accelerate the vulcanization process. Following vulcanization of the inner matrix, the tubing is cut to desired lengths and Me ends sealed with " silicone. The preferred lengths for use in connection with the present invention are about 0.5 cm. After the end seals have polymerized, the resulting capsules can either be stored, as is, or autoclived for 15 minutes on a fast exhaust cycle and stored indefinitely in a sterile form. Prior to use the capsules may"be equilibrated to establish a stable diffusion gradientacross the membrane, or used directly without equilibration.

Mother formulation for a much lower release rate is to enclose crystals of a desired substance suspended in a liquid such as water or silicone oil in a relatively nonpermeable "Ing such as Nylon-11. The release rate from this reservoir can Men be regulated 0 drilling various size hdles in Me tubing and glueing a silicone window over Me hole with silicone medical adhesive.

once again Me capsules can be sterilized by autoclaving and stored dry until use.

An exemplary technique employed by the inventors for preparing slow release hormone capsules is as follows:

Two grams of Dow Corning MX-4-4210 medical grade elastomer and 0.2 grams of Dow Corning MDX-4-4210 curing agent were weighed into a 10 ml syringe, Me bottom of which was capped with a plastic cap.

2. Six-hundred mg of 2,4-D (or NAA), from which lumps have been removed by sieving through a 411p stainless steel sieve, was added to Me V same syringe and thoroughly mixed with the Kast.omer and curing agent.

7 3. The 10 ml syringe and its contents were then degassed for 1/2 hr in a vacuum centrifuge to A remove bubbles.

Dow Corning Silastic medical grade silicone tubing (0.062" ID x 0.125" OD) of medium durometer (50 Shore A) was preswelled 10 to 30 minutes by soaking in acetone.

5. The plastic cap was removed from Me end of the ml syringe and the degassed silicone-2,4-D mixture was extruded into the preswollen tubing from which excess acetone had been removed by blowing a stream of air briefly through it.

6. Both ends of the filled tubing were then clamped shut and the tubing heated at 50 degrees (the boiling point of acetone = 56.5 degrees) overnight to accelerate the polymerization.

7. The tubing was then cut into 0.5 cm lengths.

8. The ends of the tubing sections were sealed with Dow Corning Type A medical adhesive and allowed to dry for 24 hr.

9. The finished capsules are autoclaved dry for 15-20 min and stored dry until use.

10. Before use the capsules may be preequilibrated for 48 hr by shaking in 25 ml of sterile I to W KHCO V or added to cultures without equilibration.

D. Cryopreservation Methods Cryopreservation is imp ortant because it allows one to maintain and preserve a cell culture for future use.

Cell sus pensions were cryopreserved using modifications of methods previously reported (15,49). The cryopreservation protocol comprised adding a pre-cooled (OOC) concentrated cryoprotectant mixture dropwise over a period of one hour while stirring Me cell suspension, which was also maintained at OOC during this period. The volume of added cryoprotectant was equal to the initial volume of Me cell suspension (1:1 addition), and the final concentration of cryoprotectant additives was 10% dimethyl sulfoxide, 10% polyethylene glycol (6000 M), 0.23 M proline and 0.23 M glucose. The mixture was allowed to equilibrate at OOC for 30 minutes, Wring which time-the cell suspension/ cryoprotectant mixture was divided into 1.5 ml aliquot (0.5 ml packed cell volume) in 2 ml polyethylene cryo-vials. The tubes were cooled at 0.50C/minute to -80C and held at this t emp era tur e for ice nucleation.

Once extracellular ice formation had been visually confirmed, Me tubes were cooled at 0.51C/minute from -8 to -350C. They were held at this temperature for 45 minutes (to insure uniform freeze-induced dehydration throughout Me cell clusters). At this point, the cells had lost Me majority of their osmotic volume (i.e. there is little free water left in the cells), and they could be safely plunged into liquid nitrogen for storage. The paucity of free water remaining in the cells in conjunction with Me rapid cooling rates from -35 to - 1960C prevented large organized ice crystals from forming in Me cells. The cells are stored in liquid nitrogen, which effectively immobilizes the cells and slows metabolic processes to Me point "ere long-term storage should not be detrimental.

Thawing of Me extracellular solution was accomplish ed 0 removing the cryo-tube from liquid nitrogen and swirling it in ster~le 420C water for approximately 2 minutes. The tube was removed from the heat immediately after the last ice crystals had melted to prevent heating the tissue. The cell suspension (still in the cryoprotectant mixture) was pipetted onto a filter, resting on a layer of agarose-immobilized BMS cells (the feeder layer which provided a nurse effect during recovery). Dilution of the cryoprotectant occurred slowly as the solutes diffused away through the filter and nutrients diffused upward to the recovering cells. once subsequent growth of the thawed cells was noted, the growing tissue was transferred to fresh culture medium. The cell clusters were transferred back into liquid suspension medium as soon as sufficient cell mass had been regained (usually within 1 to 2 weeks).

After the culture was reestablished in liquid (within 1 to 2 additional weeks), it was used for transformation experiments. Men necessary, previously cryopreserved cultures may be frozen again for storage.

E. DNA Seggents Comprising Exogenous Genes As mentioned previously, there are several methods to construct the DNA segments carrying DNA into a host cell that are well known to those skilled in the art The general construct of the vectors used herein are plasmids comprising a promoter, other regulatory regions, structural genes, and a 31 end.

DNA segments encoding the bar gene were constructed into a plasmid, termed pDPG165, which was used to introduce the bialaphos resistance gene into recipient cells (see Figures 1A and J. The bar gene was cloned from Streptomyces hyMscojcus (53) and exists as a 559- bp Sma I fragment in plasmid p=101. The sequence of the coding region of this gene is identical to that " 91/02071 PCr/US90/=62

published (45). To create plasmid pDPG165, the Sma I fragment -from pIJ4104 was ligated into a pUC19-based vector contaQing Me Caulif lower Mosaic Virus (CaMV) 35S promoter (derived from pB1221.1. provided by R.

Jefferson, Plant Breeding Institute, Cambridge, England), a polylinker, and the transcript 7 (Tr7) 31 end from ~Wcten= tumefaWens (31 end provided by D. Stalker, Calgene, Inc., Davis, CA).

An additional vector encoding GUS, pDPG208, (Figures 1B and D) was used in these experiments. It was const"cted using a 2.1 kb BamHI/EcoRI fragment from pAGUS1 (ptovided by J. Skuzeski, University of Utah, Salt Lake City, UT) containing Me coding sequence for GUS and Me nos 31-end from "bacteNum tumeMciens. In pAGUS1 the 51-noncoding and 51-coding sequences for GUS were modified to incorporate Me Kozak consensus sequence (24) and to introduce a new HindIII restriction site 6 bp into Me coding region of Me gene (see Figure 1E) . The 2. 1 M BamHI/EcoRI fragment from pAGUS1 was ligated into a 3.6 M BamHI/EcoRI fragment of a pUC19-based vector pCEV1 (provided Calgene,: Inc., Davis, CA) . The 3. 6 kb fragment from pCEVI contains pUC19 and a 430 bp 35S promoter from Muliflower mosaic virus adjacent to the first intron from maize AW1.

in terms W an R gene complex for use in connection with the present invention, the most preferred vectors contain Me 35S promoter from Cauliflower mosaic virus, the first intron from maize Adhl, Me Kozak consensus seque=e,-Sn:bol3 cDM, and Me transcript 7 31 end from ~Wt"um tumefaciens. one such vector prepared by Me invent"s is termed pDPG237. To prepare pDPG237 (see FiWe 1F)ythe cDNA clone of Sn:bol3 was obtained from S. Dellaporta-(Yale University, USA)4 A genomic clone of Sn was isolated from genomic DW of Sn:bol3 which had been digeWd to completion with HindIII, ligated to I lambda arms and packaged in vitro. Plaques hybridizing to two regions of cloned R alleles, R-nj and R-sc (54) were analyzed by restriction digest. A 2 kb Sst-HincII fragment from the pSn7.0 was used to screen a cDNA library established in lambda from WA of light- irradiated scutellar nodes of Sn:bol3. The sequence and a restriction map of the cDNA clone was established.

The cDNA clone was inserted into the same plant expression vector described for pDPG165, the bar expression vector (see above), and contains the 35S Cauliflower mosaic virus promoter, a polylinker and the transcript 7 31 end from A"acteAum tumefaciens. This plasmid, pPDG232, was made by inserting the cDNA clone into the polylinker region; a restriction map of pDPG232 is shown in Figure 1G. The preferred vector, pDPG237, was made by removing the cDNA clone and Tr7 31 end from pDPG232, with Aval and EcoRI and ligating it with a BamHI/EcoRI fragment from pDPG208. The ligation was done in the presence of a BaWI linker as follows:

GATCCGTCGACCATGGCGCTTCAAGCTTC GCAGCTGGTACCGCGAAGTTCGAAGGGCT The final construct of pDPG237 contained a Califlower mosaic virus 35S promoter, the first intron of Adhi, Kozak conscensus sequence, the BaWI linker, cDNA of Sn:Bol3, and the Tr7 31 end and is shown in Figure 1F.

Additional vectors have been prepared using standard genetic engineering techniques. For example, a vector, designated pDPG128, has been constructed to include the neo coding sequence (neomycin phosphotransferase (APH(31)-II)). Plasmid pDPG128 contains the 35S promoter from CaW, the neomycin phosphotransferase gene from Tn5 (66) and the Tr7 terminator from AnWacteaum tumefaMens. Another vector, pDPG154, incorporates the crystal toxin gene and was also prepared by standard techniques. Plasmid pDPG154 contains the 35S promoter, Me "ire coding region of the crystal toxin protein of MOW= M"n#ensls var. kurstaki HD 263, and the Tr7 promoter.

Various tandem vectors have also been prepared. For example, a bar/aroA tandem vector was constructed by ligating a blunt-ended 3,2 kb DM fragment containing a mutant EPSP synthase aroA expression unit (64) to NdeI- cut pDPG165 "at had been blunted and dephosphorylated (NdeI introduces a unique restriction cut approximately 200 bp downstream of the Tr7 31-end of the bar expression unit).

Transformants having aroA in both orientations relative to bar were identified.

F. Preferred Methods of Delivering = to Cells A preferred D= delivery system that does not require protoplast isolation or introduction of Aqrobacterim DM is microprojectile bombardment (8,23).

There are several potential cellular targets for microprojectile bombardment to produce fertile transgenic plants: pollen, microspores, meristems, and cultured embryogenic cells are but asfew examples. Germline transformation in maize has not been previously reported 0 bombardment of any of these types.

one of Me newly merging techniques for the introduction.of exogenous " constructs into plant cells involves Me use of microprojectile bombardment. The details of this technique and its use to introduce exogenous DW into various plant cells are discussed in Klein, 1989, Wang, et al., 1988 and Christou, et al., 1988 (22,50,8), -one method of determining the efficiency of DM delivery into Me cells via microprojectile bomba"nt employs detection of transient expression of the enzyme P-glucuronidase (GUS) in bombarded cells. For this method, plant cells are bombarded with a DM construct which directs the synthesis of the GUS enzyme.

I Apparati are available which perform microprojectile bombardment. A commercially available source is an apparatus made by Biolistics, Inc. (now DuPont), but other microprojectile or acceleration methods are within the scope of this invention. Of course, other "gene guns" may be used to introduce DM into cells.

Several modifications of the microprojectile bombardment method were made by the inventors. For example, stainless steel mesh screens were introduced below the stop plate of the bombardment apparatus, i.e., between the gun and the cells. Furthermore, modifications to existing techniques were developed the inventors for precipitating DM onto the microprojectiles.

ExMle 3: microproiectile Bombardment For bombardment, friable, embryogenic Type-II callus (1) was initiated from immature embryos essentially as set forth above in Examples 1 and 2. The callus was initiated and maintained on N6 medium (5) containing 2 mg/l glycine, 2.9 g/l L-proline, 100 mg/l casein hydrolysate, 13.2 mg/l dicamba or 1 mg/l 2,4-D, 20 g/l sucrose, pH 5.8, solidified with 2 g/l Gelgro (ICN Biochemicals). Suspension cultures initiated from these callus cultures were used for bombardment.

In the case of SC82, suspension culture SC82 was initiated from Type-II callus maintained in.culture for 3 months. SC82 cells (see Example 1) were grown in liquid medium for approximately 4 months prior to bombardment (see Table 2,w experiments &#0000;2). SC82 cells were also cryopreberved 5 months after suspension culture initiation, stored i frozen for 5 months, thawed and used for bombardmen:t (experiment 06).

In the case of suspension culture SC716 (see Example 2)4 it was initiated from Type-II callus maintained 5 months in culture. SC716 cells were cultured in liquid medium for 5 months, cryopreserved for 8 months, thawed, and used two months later in bombardment experiments #4 and J5. SC94 was initiated from 10 month old Type-II callus; and -Qltured in liquid medium for 5 months prior to bombardment (experiment 13).

Priot to bombardment, recently subcultured suspenhion culture cells were sieved through 1000 gm stainless steel mesh. From Me fraction of cell clusters passing through We sieve, approximately 0.5 ml packed cell volume , (OW) was pipetted onto 5 cm -sfilters (Whatman 14) and vacuum-filtered in a Buchner funnel. The filters were transferred to petri dishes containing three 7 cm filters (Whatman 14) moistened with 2.5 ml suspension culture medium.

The dish containing the filters with the immobilized cell suspensions was positioned 6 cm below the lexan plate used to stop Me nylon macroprojectile. With respect to the DNA, when more than a single plasmid was used, plasmid DNA was precipitated in an equimolar ratio onto tungsten particles (average diameter approximately 1.2 gm, GTEnlvania) using a modification of Me protocol described: 0 Klein, et al. (1987). In the modified pr6cedure, tungsten was incubated in ethanol at 65 degrees Q. for 12 hours prior to being used for precipitation. The precipitation mixture included 1.25 mg tungsten Tarticles, 25 gg plasmid DNA, 1.1 M CaC12 and 8.7 W spermidine in a total volume of 575 Q. After adding the c M onents in the above order, the mixture was vortexed at 40 C for 10 min, centrifuged (500 X G) for 5 min and 550 of supernatant was-decanted. From the remaining 25 of suspension, 1 gl aliquots were pipetted onto the macroprojectile for bombardment.

Each plate of suspension cells was bombarded twice at a vacuum of 28 inches Hg. In bombarding the embryogenic suspensions of A188 X B73 and A188 X B84, 100 pm or 1000 pm stainless steel screens were placed about 2.5 cm below the stop plate in order to increase the number of foci while decreasing their size and also to ameliorate injury to the bombarded tissue. After bombardment, the suspension cells and-the-supporting-filter-were-transferred-onto-solid-medium-or the cells were scraped from the filter and resuspended in liquid culture medium.

Cells from embryogenic suspension cultures of maize were bombarded with the bar-containing plasmid pDPG165 alone or in combination with a plasmid encoding GUS, pDPG208 (Fig. 1). In experiments in which a GUS plasmid was included, two of the filters containing bombarded cells were histochemically stained 48h post-bombardment.

The total number of foci (clusters of cells) per filter transiently expressing GUS was at least 1000. In two separate studies designed to quantitate transiently expressing cells (using an SC82 Q188 x B73) suspension culture), the mean number and standard deviation of GUS- staining foci per filter was 1472 +/- 211 and 2930 (n=3 and 4, respectively). The number of cells in individual foci that expressed GUS averaged 2-3 (range 1-). Although histochemical staining can be used to detect cells transformed with the gene encoding GUS, those cells will no longer grow and divide after staining. For detecting stable transformants and growing them further, e.g., into plants, selective systems compatible with viability are required.

G. Methods = Identifying Transformed Cells V It is believed Mat DM is introduced into only a small percentage of cells in any one experiment. In order to provide a more efficient system for identification of those cells receiving DM and integrating it into their genomes, therefore, one may desire to employ a peans for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some agent, e.g. an antibiotic or herbicide. The potentially transformed cells are Wen exposed to the agent. In the population of surviving cells me those cells wherein generally Me resistance-conferring gene has been integrated and expressed at sufficient levels to survive. Cells may be tested further to confirm stable integration of We exogenous "a- Using-embryogenic suspension cultures, stable transformants me recovered at a frequency of approximately 1 per" 1000 transiently expressing foci. A specific embodiment of this procedure is shown in Example 5.

One of-the.difficulties in cereal transformation, e.g., corn, has been the lack of an effective selective agent for transformed cells, from totipotent cultures (36). Stable transformants were recovered from bombarded nonembryMenic Black Mexican Sweet (BMS) maize suspension culture cells, using the neo gene and selection with We aminoglycoside, kanamycin (22). This approach is limited because many monocots are insensitive to hip concentrations of aminoglycosides (12,19). The stage of cell growth. duration of exposure and concentration of Me allibio tic, my be critical to Me successful use of aminoglyco,sides as selective agents to identify 4n.

transformants (26,51,52). In addition, use of the aminoglycosides, kanamycin or G418, to select stable

transformants from embryogenic maize cultures, in the inventors, experience, often results in the isolation of resistant calli that do not contain the neo gene.

one herbicide which has been suggested in resistance studies is the broad spectrum herbicide bialaphos.

Bialaphos is a tripeptide antibiotic produced by Streptomyces h~roscoacus and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. upon removal of the L- alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (33). Inhibition of GS in plants by PPT causes the rapid accumulation of amm onia and death of the plant cells.

The organism producing bialaphos also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the bar gene. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (MT) is referred to in DE 3642 829 A wherein the gene is isolated from Streptomyces-Wadochr=ogenes.

This enzyme acetylates the free amino group of PPT preventing auto-toxicity (45). The bar gene has been cloned (29,45) and expressed in transgenic tobacco, tomato and potato plants (10) and Brassica (11). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial PPT and bialaphos in greenhouses.

PCT Application No. WO 87/00141 refers to the use of a process for protecting plant cells and plants against the action of glutamine synthetase inhibitors. This application also refers to the use of such of a process WO 91102071 PCF/US90/04462 50 to develop herbicide resistance in determined plants.

The gene enmding resistance to Me herbicide BASTA (Hoechst phAphinothricin) or Herbiace (Meiji Seika bialaphos) was spid to be introduced by ~bacterium infection into tobaceo (Acotiana tabacum cv Petit Havan SRI), potato,(Solmum tuberosum cv Benolima) and tomato (Ocopersi=m esculentum) and conferred on plants resistance- to application of herbicides.

An exemplary embodiment of vectors capable of delivering DM to plant host cells is the plasmid, pDPG165. This plasmid is illustrated in Fig. 1A and IC.

A very important component of this plasmid for purposes of genetic transformation is Me bar gene which acts as a marker for selection of transformed cells.

Example 49 -Selection of bar Transformants Using Bialaphos

The suspension culture (designated SC82) used in the initial experiments (see Example 3) was derived from embryogenic Type-II callus of A188 X B73. Following bombardment (see Wample 3), cells onsfilters were resuspended in nonselective liquid medium, cultured for 1 to 2 weeks and transferred to filters overlaying solid medium containing 1 or 3 mg/l bialaphos. The degree of inhibition tissue gr"h during selection was dependehi'upon-the density of Me cells on the filter and on We concentration of bialaphos used. At the density plated (0.5 PN/filter), Me growth of the cells cultured on 1 mg/l bialaphos was only partially inhibited (-30- 50% of nonselectud growth) and after 3 to 4 weeks much of this tissue was transferred as discrete clumps (-5 mm in diametery to identical medium. On medium containing 3 mg/l bialaphos, Me growth of cells on Me original selection filter was severely inhibited (-10% of nons6lected growth) and selection was carried out without Using either selection protocol (1 or 3 mg/l bialaphos), resistant cell colonies emerged on the selection plates of SC82 bombarded with pDPG165 approximately 6 to 7 weeks after bombardment (Fig. 2A).

Bialaphos-resistant calli were maintained and expanded on selection medium. Much of this tissue was

embryogenic (Fig. 2B). No colony growth occurred on plates to which cells were added from suspension cultures on which no transforming attempts were made. These are controls which confirm the prediction that cells without the bar gene are not resistant to bialaphos.

Colonies on solid supports are visible groups of cells formed by growth and division of cells plated on such support. Colonies can be seen in Fig. 2A on a petri dish. In this figure, the cells capable of growth are those that are resistant to the presence of the herbicide bialaphos, said resistance resulting from integration and expression of the bar gene. Exposure of cells was to 1 mg/1 bialaphos. Figure 2B is a magnification showing the morphology of one bialaphos-resistant culture maintained on selection media indicating that growth is embryogenic.

As a confirmation that the cells forming the colonies shown in Fig. 2 had indeed incorporated the bar gene and were expressing it, bialaphos-resistant callus lines were analyzed for activity of the bar gene product, phosphinothricin acetyl transferase (PAT), by thin-layer chromatography. Protein extracts from eleven callus lines (EI-11) isolated from SC82 bombardment experiments contained PAT activity as shown in Figure 3 and activity levels varied approximately 10-fold among the isolates.

Still further and mote direct confirmation of the presence of the bar gene was obtained by analysis of the WO 91102071 PCT/US90/04462 removing the tissue from the original filter.

gen.ic WA of potential transf ormants P DNA gel blots (Figure 4) Me sources of DNA which were electrophoresed = ough the gel were the bialaphos- resistant callhi lines designated El-Ell and a non-selected control, M. (Fig. 1 indicates the cleavage sites of Mos-e enzymes within the bar gene plasmid.) After Me DNA was electrophoresed %rough Me gel and transferred to nylon membranes, the resulting blot was hybridized with a 32p_labeled bar gene sequence from the plasmid pDPG165. Me radioactivity used per blot was approximately 25 X 106 Cerenkov cpm. The lane in Figure 4 designated "C and "Q copies contain 1.9 and 9.5 pg respectively of Me 1.9 kb bar expression unit released from Me plasmid pDPG165 by application of the EcoRI and HindIII enzymes; Wese amounts represent about 1 and 5 copies per diploid genome.

Genomic " from all eleven bialaphos-resistant isolates contained bar-hybridizing sequences as shown in Figure 4. The hybridization in all isolates to a fragment migrating slightly larger Man 2 kb may be We to contaminating pUC19 sequences contained in this bar probe preparation; no such hybridization occurred in subsequent experiments using Me same genomic DNA and a different preparation of the bar probe. Hybridization to a 1.9 " fragment in eight of the eleven isolates indicated Wat'Wese isolates contained intact copies of Me 1.9 " Wlexpression unit. Me estimated copy numbere of Me intact unit ranged from one or two (El, E7, E8, E10, Ell) to approximately 20 (E3, E4, E6).

Hybridizatioi with We bar probe in isolates E2 and E5 occurred only to a single, higher molecular weight fragment (-3 To establish that'the PAT coding sequence was intact in isolates E2 and E5, genomic DNA was digested with SmaI, which, releases a 559 bp fragment containing the PAT structural gene (Figure 1A), and subjected to DM gel blot analysis using 32 P-labeled bar. This analysis confirmed the presence of a singly intact copy of bar.

Expression of PAT in these isolates may not be dependent on the 35S promoter or the Tr7 31 end. The hybridization patterns of some of the isolates were identical (E2 and E5; E7 and E8; E3, E4, and E6); therefore, it is probable that some isolates did not arise from independent transformation events but represent transformants that were separated during selection.

Seven hybridization patterns were unique, likely representing seven independent single-cell transformation events. The patterns and intensities of hybridization for the seven transformants were unchanged during four months in culture, providing evidence for the stability of the integrated sequences. The seven independent transformants were derived from two separate bombardment

experiments. Four independent transformants representing isolates E2/E5, E3/E4/E6, El and E7/E8, were recovered from a total of four original filters from bombardment experiment 11 and the three additional independent transformants, E9, E10, and Ell, were selected from tissue originating from six bombarded filters in experiment #2. These data are summarized in Table 2.

Table 2 Summary of Maize Transformation Experiments 2 P"Of with Independent Intact of bar bar with GUS Cointegration Coexpression E)y~ culture Filters Transform, ants Expression, Coding - GUS Frequency - FrequM B-ombarded Bombard~d Recovered Units Sequerice ~~M %) I SC82 4 4 3 rLa.

2 SC82 6 3 2 ma.

3 SC94 10 8 6 n.a.

4 SC716* 8 13 8 11 3 85 23 SC716* 8 7 4 6 1 86 14 6 SC82* 4 19 17 13 3 68 16 Totals 40 54 40 30 7 77(30/39) 18(7/39) culture reinitiated from cryopreserved cells n.a. not applicable; only pDPG165 DNA used or cotransformation analysis not done " 91/02071 PCUUS90/M62 Studies with other embryogenic suspension cultures produced similar results. Using either an SC82 culture that was reinitiated from cryopreserved cells (experiment #6) or an A188 x B84 (SC94) suspension culture (experiment Q), numerous independent transformants were recovered (19 and 18 respectively; Table 2). All transformants contained the bar gene and expressed MT.

The copy number of bar-hybridizing sequences and levels of MT expression were comparable to the studies described above.

Example 5: Integration of the Bar Gene into Cell Lines Derived from the SC716 Suspension Culture

Bombardment studies and subsequent analyses were also performed on the A188xB73 suspension culture, termed SC716 (see Example 1). The resultant transformed plant cells were analyzed for integration of bar genes. To carry out this analysis, genomic DNA was obtained from RI-R21 isolates; 6 gg of DNA was digested with the restriction endonucleases EcoRI and HindIII, and DNA gel blot analysis was performed using the bar gene as probe.

In Fig. 9, molecular weights in kb are shown to the right and left. The untransformed control is designated "W,11 and the last column to the right contains the equivalent of two copies of the bar gene expression unit per diploid genome. For the DNA load used, two copies the bar expression unit per diploid genome is 5.7 pg of the 1.9 kb EcoRI/Hind fragment from the plasmid pDPG165. The DNA separated on the gel blot was hybridized to a 32P-labeled bar probe. The label activity in the hybridization was approximately 10 X 106 Cerenkov cn. In A, We presence of an intact bar expression unit is inferred from the hybridization of the bar probe to a 1.9 kb band in the gel.

Example 6: Assays for Integration and E"ression of GUS SC716 transformants discussed in Ex amp le 5, were V further analyzed for integration and expression of the gene encoding GUS. As determined by histochemical assay, four of Me SC716 transformants (R5, R7, R16, and R21) had detectable GUS activity 3 months post-bombardment.

Expression patterns observed in Me four coexpressing callus lines varied. The number of cells with GUS activity within any given transformant sampled ranged from -5% to -90% and, in addition, the level of GUS activity within those cells varied. The cointegration frequency was determined 0 washing the genomic blot hybridized with bw (Figure 9A) and probing with 32 P- labeled GUS sequence as shown in Figure 9B. EcoRI and HindIII, which excise Me bw expression unit from pDPG165, also release from pDPG208 a 2.1 kb fragment containing Me GUS coding sequence and the nos 31 end (Figure 1B).- Seventeen of We independent bar transformants contained sequences "at hybridized to the GUS probe; three, R2, RA and R19 did not. Transformants in which GUS activity wasdetected (R5, R7, R16 and

R21) had intact copies of We 2.1 kb EcoRI/HindIII fragment containing Me GUS structural gene (Figure 9B).

Transformant,s Mat contained large numbdrs of fragments that hybridized to bw (R1, R5, R21) also contained comparable number of fragments that hybridized to the gene encoding GUS (Figures 9A and B). This observation is consistent with those reported using independent plasmidsAfi PEG mediated transformation of A188 X MS protoplasts (Lyznik, et al., 1989) and in studies conducted a We inventors involving bombardment-mediated transformation of MS suspension cells.

Co-Transformation Co-transformation may be achieved using a vector containing the marker and another gene or genes of interest. Alternatively, different vectors, e.g., plasmids, may contain the different genes of interest, and the plasmids may be concurrently delivered to the recipient cells. Using this method, the assumption is made that a certain percentage of cells in which the marker has been introduced, have also received the other gene(s) of interest. As can be seen in the following examples, not all cells selected by means of the marker, will express the other genes of interest which had been presented to'the cells concurrently. For instance, in-Ex-amp le-7,-successful cotransformation occurred in 17/20 independent transformants (see Table 2), coexpression occurred in 4/20. In some transformants, there was variable expression among transformed cells.

Example 7: Co-Integration and Co-Expression of the Bar Gene and the GUS Gene to Cell Lines Derived from the SC82 SusRension culture Of the bialaphos-resistant isolates selected from a reinitiation of cryopreserved SC82 cells transformed with separate plasmids (as described for SC716), nineteen independent transformants were selected in this experiment (experiment #6, Table 2). The frequency of cointegration and coexpression in those isolates was similar to that described for SC716 isolates (Table 2).

The pattern of GUS staining in these transformants varied in a manner similar to that described for coexpressing SC716 transformants. A transformant, Y13, which contained intact GUS coding sequence, exhibited varying levels of GUS activity as show in Figure 8. This type of expression pattern has been described previously in cotransformed MS cells (Klein, et al., 1989). Variable " 91 M2071 PMUS90/"2 activity detected in Me cells from a single transformant may be attributed to unequal penetration of the WS substrate, or differential enression, methylation, or Me absence of Me gene in some cells.

These,results show that both the bw gene and Me WS gene me present in some of the cells bombarded with We two plasmids containing these genes. Co- transformation has occurred. In Me cotransformation examples described herein and summarized in Table 2, cotransf"ation frequency of We non-selected gene was 77%; coenrypsion frequency was 18%.

1. Regeneration of Plants From Transformed Cells

For use~ih,agriculture, transformation of cells "ro is only one step toward commercial utilization of these new methods. Plants must be regenerated from the transformed cells, and Me regenerated plants must be jo developed i nto full plants capable of growing crops in open &Md For this purpose, fertile corn plants are required.- Th invention disclosed herein is the first successful production of fertile maize plants (e.g., see Figure 1M) from transformed cells.

one efficient regeneration system involves transfer of embryogenic callus to MS (Mrashige and Skoog, 1962) medium -containing 0.25 mg/1 2;4-dichlorophenonacetic acid and 10.0 mg/l 6-benzyl-minopurine. Tissue was maintaine& on this medium for approximately 2 weeks and subsequently transferred to MS medium without hormones (Shillito, et al., 1989). Shoots that developed after 2- 4 weeks on, hormonp-free medium were transferred to MS medium containing 1% sucrose and solidified with 2 g/l GelgroR in Plant Co p containers "ere rooting occurred.

Another successful regeneration scheme involved transfer of embryogenic callus to N6 (Chu, et al., 1975) medium containing 6% sucrose and no hormones (Armstrong and Green, 1985) for two weeks followed by transfer to MS medium without hormones as described above. Regeneration was performed at 250C under fluorescent lights (250 microeinsteins . M-2 -s-1). After approximately 2 weeks developing plantlets were transferred to soil, hardened off in a growth chamber (85% relative humidity, 600 pp:

pm COV 250 microeinsteins*M-2.S-1) , and grown to maturity either in a growth chamber or the greenhouse.

Regeneration of plants from transformed cells requires careful attention to details of tissue culture techniques. one of the major factors is the choice of tissue culture media. Mere are many media which will support growth of plant cells in suspension cultures, but some media give better growth than others at different stages of development. Moreover, different cell lines respond to specific media in different ways. A further complication is that treatment of cells from callus initiation through transformation and ultimately to the greenhouse as plants, requires a multivariate approach.

A progression consisting of various media types, representing sequential use of different media, is needed to optimize the proportion of transformed plants that result from each cell line. Table 3 illustrates sequential application of combinations of tissue culture media to cells at different stages of development.

Successful progress is ascertained 0 the total number of plants regenerated.

REGENERAITON MEDIA PROGRESSIONS 0 227b 227b 227b 227b 227b 227b 227b 227b 227b 201b 52 163 205 227b 201b 205 163 227b 201b PLANTS 227b 171 171 171 171 171 173 173 173 173 177 177 TO Cell Line 101 101 101 101 101 101 101 101 101 101 101 101 SOIL 19 CONTROLS AOIC-11 x 4 X x x X .2 x x x x x 6* AOIC-01 N 7 x x I X Z7 _ _ , X X - X x 34* TOTAL x 11 x x x X 29 X X~% "X x x 40* TRANSFORMED AOIC-11 x X, ~ x 0 0 0 x '. x 0 0 x x 0 AOIC-12 x 2 x 011-1 0 0 x x 0 0 x x 2 AOIC43 x 5 1 4 0 0 1 1 1 1 x x 14* A01C-14 x 2 x 0 0 0 x x 1 0 x x 3* A01C-15 X 28 0 12 7 1 23 13 0 0 0 0 84* A01C-17 x 7 0 0 0 0 17 0 0 0 0 0 24 A01C,18 x 12 0 0 x 0 21 10 0 x 2 0 45* AOIC49 x 0 x x 0 x 0 x x 0 x 0 0 AOIC-20 x 10 x 0 0 x 0 x x 0 x 0 10* A01C-21 x 0 x x x x 0 x x x x 0 0 A01C-24 2 4 0 0 0 0 6 5 0 0 0 0 17* AOIC-25 x 9 x x 0 0 1 x 0 0 x x 10 A01CA7 x 0 x x x x 10 x x x x 0 10* TOTAL 2 1 16 7 1 79 29 2 1 2 0 219* COMBINED

CONTROLS x 11 x x x x 29 x x x x x 40* TRANSFORMED 2 79 1 16 7 1 79 29 2 1 2 0 219* TOM 2 90 1 16 7 1 108 29 2 1 2 0 259* X Regeneration not attempted by this route. 201b 1 mg/1 bialophos.

* Mon Oants could have been taken to soil. 227b = 227 with 1 mg/I bialophos.

WO 91102071 PGr/US90104462 It can be seen that using the same group of media, cell lines will vary in their success rates (number of plants) (Table 3). There was also variation in overall success rate, line A01-15 yielding the greatest number of plants overall. (It should be noted, however, that because tissue was limiting not all combinations of media were used on all lines, therefore, overall comparisons are limited.) A preferred embodiment for use on cell lines in general, at least initially, is the combination show in the second column under the regeneration media progression (media 227, 171, 101, 501). Media 227 is a good media for the selective part of the experiments, for example, to use for growth of callus in the presence of bialaphos. This media contains the hormone dicamba. NAA and 2,4-D are hormones in other media. In liquid media, these are usually encapsulated for controlled release (see Example 12 hereinbelow).

Thus, it can be seen from Table 1 that the various media are modified so as to make them particularly applicable to the development of the transformed plant at the various stages of the transformation

process. For example, subculture of cells in media 171 after applying the selective agent, yields very.small embryos.

Moreover, it is believed that the presence of BAP in the media facilitates development of shoots. Myo-inositol is believed to be useful in cell wall synthesis. Shoot elongation and root development proceeds after transfer to media 101. 101 and 501 do not contain the hormones that are required for earlier stages of regeneration.

Transfer of regenerating plants is preferably completed in an agar-solidified media adapted from a nutrient solution developed by Clark (1982; ref. 6), media 501. The composition of this media facilitates the hardening of ME-developing plants so that they can be transferred to We greenhouse for final growth as a plant! The salt concentration of this media is significantly different from that of the three media used in Me earlier stages, forcing the plant to develop its own metabolic pathways. These steps toward independent growth are required before plants can be transferred from tissue culture vessels (e.g. petri dishes, plant cans) to Me greenhouse.

Approximately 50% of transformed callus lines derived from Me initial SC82 and SC716 experiments were regenerable 0 Me routes tested. Transgenic plants were regenerated from four of seven independent SC82 transf orm ants and ten of twenty independent SC716 transformants.

Regeneration of thirteen independently, transformed cell lines and two control lines of SC716 was pursued.

Regeneration was successful from ten of thirteen transformants. Although a total of 458 plantlets were regenerated, We tq,time and space constraints only 219 transformednplants~(representing approximately 48% of the total number-o 'sfregenerants) were transferred to a soilless mix (We below). Approximately 185 plants survived. Twelve; regeneration protocols were investigated,and the number of plants regenerated from each route has been quantified (Table 3). There appeared to be no significant advantage to maturing the tissues on 201, 52, 163, or 205 (see Table 1 for media codes) prior to transfer to medium 171 or 173. The majority of the plants were generated by subculturing embryogenic callus directly from 227 to either 171 or 173. These plantlets developed roots without addition of exogenous auxins, and plantlets were thenl transferred to a soilless mix, as was necessary for on of Me transformants regenerated from SC82.

I V The soilless mix employed comprised Pro Mix, Micromax, Osmocote 14-14-14 and vermiculite. Pro Mix is a commercial product used to increase fertility and porosity as well as reduce the weight of the mixture.

This is the bulk material in the mixture. Osmocote is another commercial product that is a slow release fertilizer with a nitrogen-phosphorus-potassium ratio of 14:14:14. Micromax is another commercial fertilizer that contains all of the essential micronutrients. The ratio used to prepare the soilless mix was: 3 bales (3 ft 3 each) Pro Mix; 10 gallons (vol.) vermiculite; 7 pounds Osmocote; 46 ml Micromax. The soilless mix may be supplemented with one or two applications of soluble Fe to reduce interveinal chlorosis during early seedling and plant growth.

Regeneration of transformed SC82 selected cell lines yielded 76 plants transferred to the soilless mix, and 73 survived. Me plants were regenerated from six bialaphos-resistant isolates, representing four of seven clonally independent transformants. Eighteen protocols were used successfully to regenerate the seventy six plants (Table 4). Differences in morphology between cell lines deemed some protocols more suitable than others for regeneration.

TABLE 4 EFFECTS OF PROGRESSION OF MEDIA ON " NIM1WR OF PLANTS ~NERATE[) 22n 22M MM M711 MM 227B 227B 227B 227B 227B 227B 227B 201B 227B 201B 201B 201B 201B

227B 227B 227B 227A 227A 227A 171 52 52 52 171 201B 227B 205 227B 205 1 52 OF 142 173 171 205 209 173 173 473, 173 171 173 , 473, 178 171 177 177 178 171PLANTS CELL 101 101 101 101 101 101 101 101 101 101 101 101 101 101 101 101 101 101 LINE 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 501 113-14-4 1 X 14 X x x I I x 2 x X 5 X 5 x x x 29 113-14-9 X X 1 1 X 4 1 x x x x x x x I x I x x 9 133-14-7 X X x x x x x x x x x 6 2 x x x x x 1 9 113-14-6 X X X X 1 x x x x x x x x x x x x x x I 113-14-3 X X x x x x x x x x x x x x x x x x x x 0 113-14-2 X X x x x x x x x x x x x x x x x x x 0 133-14-1 X X x x x x x x x x x x x x x x x x x 0 133-14-5 X X X X X x x x x x x x x x x x x x 0 133-13-5 X X X X X X X X X X x x x x x x x x x 0 113-13-2 X 1 13 X X X 3 2 2 x x x x x 1 X X X 22 133-13-1 X 3 x I x x x x I x x x x x x x x I X 6 TOTAL 1 4 28 2 1 4 5 3 3 2 6 2 5 1 6 1 1 1 76 * = See table 1 for media codes. ct 1.11 X = This media progression was either attempted and unsuccessful or not attempted.

227 with 1C M M.

227B 227 with 1 mg/1 bialaphos.

WO 91102071 PCr/US90/04462 Prior to regeneration, the callus was transferred to either a) an N6-based medium containing either dicamba or 2,4-D or b) an MS-based medium containing 2,4M. These steps allowed further embryoid development prior to maturation. Most of the maturation media contained high BAP level, J-10mg/1) to enhance shoot development and cause proliferation. An MS-based medium with low 2,4-D (0.25 mg/l) and high BAP (10 mg/1), as described by Shillito, et al., 1989, was found to be quite effective for regeneration.

Likewise, an MS-based medium containing Igm NAA, 1 pm IM, 2 gm 2-IP, and 5 mg/l BAP (modified from Congar, et al., 1987) also promoted plant regeneration of these transformants. After plantlets recovered by any of the regenerative protocols had grown to five cm, they were transferred to a nutrient solution described by Clark, 1982, which was solidified with Gelgro. Plantlets which were slow to develop roots were treated with 3 Q droplets of 0.3% IBA at the base of the shoot to stimulate rooting. Plants with well developed root systems were transferred to a soilless mix and grown in controlled environmental chambers from 5-10 days, prior to transfer to the greenhouse.

J. Assays for Integration of Exogenous DW and Expression of DNA in Rn _R,_Plant.

Studies were undertaken to determine the expression 00 of the transf ormed gene (s) in transgenic , and R, plants.

Functional activity of MT was assessed by localized application of a commercial herbicide formulation containing PPT to leaves of SC82 RO and R, plants. No necrosis was observed on leaves of & plants containing either high levels (E2/E5), or low levels (E3/E4) of MT.

Herbicide-treated E3/E4/E6 and control leaves are show in Figure 10A. Herbicide was also applied to leaves of E2/E5 progeny.segregating for bar. As demonstrated in Figure 10B, Saves of R, plants expressing bar exhibited no necrosis Edx days after application of the herbicide while R, plants without bar developed necrotic lesions.

No necrosis was observed on transformed leaves up to 30 Mys post"plication.

.4.

Twenty-bhe plants, representing each of the four regenerable transformed SC82 callus lines, were also analyzed for.expression of Me bar gene product, PAT, Min-layer chromatographic techniques. Protein extracts from We lhavea of Me plants were tested. PAT activity of one plant regenerated from each callus line is shown in Fig. 5.

All 21 plants tested contained PAT activity.

Furthermore, activity levels were comparable to levels in Me callus lines from which the plants were regenerated.

Me nontransformed plant showed no PAT activity (no band is in Me expected position for acetylated PPT in Me autoradiograph from We PAT chromatogram). A band appears in Me BMS lane Mat is not in lanes containing protein extralct1from the plant leaves. This extra band was believed to be an artifact.

As another method of confirming that genes had been delivered toVells and integrated, genomic (chromosomal) DNA was isolated from a nontransformed plant, the four regenerable callus lines and from two n plants derived from each chiludline. Figure 6 illustrates results of gel blot analysjs of genomic DNA from the four t transformed dalli (C) and Me n plants derived from them. The transformed callus and all plants regenerated from transformed callus contained sequences that hybridized to Me bar probe, indicating the presence of DO sequences, Mat were complementary to bar.

Furthermore,An all instances, hybridization patterns A ~' observed in plant DNA were identical in pattern and intensity to the hybridization profiles of the corresponding callus DNA.

DNA from E3/E4/E6 callus and the desired 0 plants contained approximately twenty intact copies of the 1.9 kb bar expression unit (Cauliflower Mosaic Virus 35S promoter-bar-Q"acWrW transcript 7 31-end) as well as numerous other bar-hybridizing fragments. Ell callus and plant DNA contained 1-2 copies of the intact expression unit and 5-6 additional non-intact hybridizing fragments. Elo callus and plants contained 1-2 copies of the intact bar expression unit. E2/E5 DNA contained a single fragment of approximately 3 kb that hybridized to the probe. To confirm that the hybridizing sequence observed in all plants were integrated into the chromosomal DNA, undigested genomic DNA from one plant derived from each independent transformant was analyzed by DNA gel blot hybridization. Hybridization to bar was observed only in high molecular weight DNA providing evidence for the integration of bar into the maize genome.

Plants were regenerated from the coexpressing callus line, Y13, shown in Figure 8. Plants regenerated from Y13 (experiment #6, Table 2) were assayed for GUS activity and histochemically stained leaf tissue from one plant is shown in Figures 10C, D, E. Numerous cell types including epidermal, guard, mesophyll and bundle sheath cells stained positive for GUS activity. Staining intensity was greatest in the vascular bundles. Although all leaf samples from the regenerated plants tested (5/5) expressed the nonselected gene, some non-expressing leaf sectors were also observed. Leaf tissue extracts from three Y13 and three control plants were also assayed for GUS activity by fluorometric analysis (Jefferson, 1987).

Activity detected in two opposing leaves from each of three Y13 plants-tested was at least 100-fold higher than Mat in control leaves.

I =ample 8: General Methods for Assays

Z A method to detect the presence of phosphinothricin acetyl transferase (PAT) activity is to use thin layer chromatography.

An, example of such detection is shown in Fig. 5 wherein-various protein extracts prepared from homogenates of potentially transformed cells, and from control cells that-have neither been transformed nor exposed-to bialaphos selection, are assayed by incubation with PW and 14C-Acetyl Coenzyme A., 25 gg of protein extract were loaded per lane. The source in lanes El- Ell were SC82 transfQrmants; B13 is a BMS (Black Mexican Sweet corn nonembryogenic) bar transformant. EO is a nonselected, nontransformed control.

AS can be seen at Me position indicated by the arrow (Me position expected for the mobility of 14C-N-AcPPT), all lanes except Me nontransformed control have activities with Me appropriate mobility. Variation in activity among Me transformants was approximately 10 fold, as demonstrated 0 the relative intensity of the bands.7 Me results of this assay provide confirmation of We expression of Me bar gene which codes for PAT. For analysIs of " activity in plant tissue, 100-200 mg of leaf tissue was extracted in sintered glass homogenizers and assayed as described previously.

MS activity was assessed histochemically as described using 5-Momo-4-chloro-3-indolyl glucuronide (Jefferson, 1987); tissue was scored for blue cells 18- 24 h after addition of substrate. Fluorometric analysis was performed as described a Jefferson (1987) using 4- methyl umbelliferyl glucuronide.

DNA gel blot analysis was performed as follows.

Genomic DNA was isolated using a procedure modified from Shure, et al., 1983. Approximately 1 gm callus tissue was ground to a fine powder in liquid N2 using a mortar and pestle. Powdered tissue was mixed thoroughly with 4 ml extraction buffer (7.0 M urea, 0.35 M NaCl, 0.05 M Tris-HC1 pH 8.0, 0.01 M EDTA, 1% sarcosine).

Tissue/buffer homogenate was extracted with 4 ml phenol/ chloroform. The aqueous phase was separated by centrifugation, passed through Miracloth, and precipitated twice using 1/10 volume of 4.4 M ammonium acetate, pH 5.2 and an equal volume of isopropanol. The precipitate was washed with 70% ethanol and resuspended in 200-500 Q TE (0.01 M Tris-HC1, 0.001 M EDTA, pH 8.0).

Plant tissue may also be M loyed for the isolation of DNA using the foregoing procedure.

Genomic DNA was digested with a 3-fold excess of restriction enzymes, electrophoresed through 0.8% agarose (MC), and transferred (Southern, 1975) to Nytran (Schleicher and Schuell) using 1OX SCP (20X SCP: 2 M NaCl, 0.6 M disodium phosphate, 0.02 M disodium EDTA).

Filters were prehybridized at 650C in 6X SCP, 10% dextran sulfate, 2% sarcosine, and 500 gg/ml heparin (Chomet, et al., 1987) for 15 min. FAters were hybridized overnight at 650C in 6X SCP containing 100 pg/ml denatured salmon sperm DNA and 32P-labeled probe. The 0. 6 kb SmaI fragment from pDPG165 and the 1.8 kb BamHI/EcoRI fragment from pCW5 were used in random priming reactions (Feinberg and Vogelstein, 1983; Boehringer-Mannheim) to generate labeled probes for detecting sequences encoding PAT or GUS, respectively. Filters were washed in 2X SCP, 1% SDS at 650C for 30 min. and visualized by autoradiography using Kodak " 5 film. Prior to rehybridization with a second probe, the filters were boiled for 10 min. in distilled H20 to remove the first probe and then prehybridized as described above.

Example 9-: '~' , Herbicide ~lication

- The herbicide formulation used, Basta TX R , contains 200 g/l glufosinite, Me ammonium salt of phosphino"icin. Young leaves were painted with a 2% Basta solution (v/v) containing 0.1% (v/v) Tween-20. The prescribed application rate for this formulation is 0.5- In Figure 10A, BastaR solution was applied to a large area (about 4 k 8 cm) in the center of leaves of a nontransformed A188 X B73 plant (left) and a transgenic RD E3/E4/E6 plant (right). In Figure 10B, Basta was also applied to leaves of J our R, plants; two plants without bw and two plants containing bar. The herbicide was applied to R- plants in 1 cm circles to four locations on each leaf, two on each side of We midrib. Photographs Z were taken sly days after application.

K. FertiliQ of Transgenic Plants To recover progeny the regenerated, genetically transformed maize plants (designated Q , were backcrossed with pollen collected from nontransformed plants derived from seeds, and progeny (designated Rj) M at contained and expressed bar were recovered.

m important aspect of Ws invention is the production for M first time of fertile, genetically M . ~, transf ormed Cize plants (S)_ and progeny (R,) . These were regenCated from embryogenic cells that were Mansfomed,'-Rl,plants are those resulting from backcrossing of S plants.

Pollination of transgenic S ears with non- transformed B73 pollen resulted in kernel development.

In addition, kernels developed from pistillate flowers on male inflorescences that were pollinated with non-.transformed B73 pollen. Kernels on transformed S plants from SC82 developed normally for approximately 10-14 days post-pollination but after this period the kernels ceased development and often collapsed. Most plants exhibited premature senescence at this time. A total of 153 kernels developed sporadically on numerous plants (see Table j: 8 of 37 E2/E5 plants, 2 of 22 E10 plants, and 3 of 6 Ell plants. Viable progeny were recovered by embryo rescue from 11 E2/E5 plants and one E10 plant.

. i Table 5. Regenerated Plants (RJ and progeny (RJ kS of Independent of bar Regenerable &#0000; of R. &#0000; of RV Culture Transformants Transformed R. Reaching Producing Kernels R, Bombarded Recovered Callus Unes Kernels Rtco-v&r-e-d Plants 1,2 SC82 7 4 76 73 23 153 40 4,5 SC716 20 10 219 (35) (9) (51) (31) 3 SC94 8 2* ill (0) (0) (0) (0) 6 SC82 19 4* 23* (0) (0) (0) (0) " Regeneration in progress.

0 Experiment still in progress, data still being collected.

WO 91102071 PCF/US90/04462 derived B73 plants. To date, from the 35 mature SC716 plants nine plants (representing four independent callus lines) yielded 51 kernels, 31 of which produced vigorous R, seedlings (Table 5). Most kernels that developed on SC716 plants did not require embryo rescue. Kernels often developed for 30-40 days on the plant and some were geminated in soil. The remaining seed was geminated on MS-based medium to monitor gemination and transferred to soil after a few days. In addition to the improved kernel development observed on SC716 S plants relative to SC82 Ro plants, pollen dehisced from anthers of several SC716 plants and some of this pollen germinated in At.ro (Pfahler 1967). Transmission of the foreign is gene has occurred both through SC716 R, ears and using SC716 Rpderived pollen on non-transformed ears.

Pollen obtained from transformed R, plants has now been successfully emp loyed to pollinate B73 ears and a large number of seeds have been recovered (see Figure 11j. Moreover, a transformed ear from an R, plant crossed with pollen from a non-transformed inbred plant is shown in Figure 11D. The fertility characteristics of the R, generation has been confirmed both from a standpoint of the pollen's ability to fertilize non-.transformed- ears, and the ability of R, ears to be fertilized by pollen from non-transformed plants.

ExMle 10: Analysis of Progeny (RQ of Transformed R. Plants for PAT and bar A total of 40 progeny of E2/E5 0 plants were analyzed for PAT activity, ten of which are show in Figure 7A. Of 36 progeny which were assayed, 18 had PAT activity. Genomic DM from the same ten progeny analyzed for PAT activity was analyzed by DM gel blot hybridization for We presence of bar as shown in Figure 7B. M six progeny tested Mat expressed " contained a single c, opy of bar identical in nobility to that

. detected in mllus and , plants; thesfour MT-negative progeny tested -did.not contain bar-hybridizing sequences.

In one serim of assays, the presence of the bar gene product in 18-of,36 progeny indicates a 1:1 segregation of We singletcopy ofibar found in E2/E5 , plants and is consistent with inheritance of PAT expression as a single dominant trait.* A dominant pattern of inheritance would indicate ti~e presence in the plant of at least one copy of Me genev=ding for ". The single progeny recovered from an E10 0 plant tested positive for PAT activity.

It.was determined Mat the methods disclosed in this invention resulted in Yansf ormed RO and R, plants that produced functionally active This was determined by applying Basta (PPT) to Me leaves of plants and determining whether necrosis (tissue destructiony- resulted from this application. If functionally active 2 0 is produced by We plants, the leaf tissue is protected fry necrosis. No necrosis was observed on RO plants hip levels of MT (EV/E5) or on plants expressing low levels (E3/E4/E6) (Fig. 10A).

Herbicide was also applied to leaves of R, progeny segregating for bar. In these studies, no necrosis was observed on Rlplants containing and expressing bar, however, necrosis was observed on those R, plants lacking the bar gene. -This is shown in Fig 10B.

Segregation of bar did not correlate with the variability in.phenotypic characteristics of R, plants such as plant Adight and: tassel morphology. In Figure 9B, Me planj:on the right contains bar, the plant on the Aeft does pot. In addition, most of the R, plants were more vigorous Man the 0 plants.

Of the 23 R, seedlings recovered to date from the SC716 transformants, ten of 16 had PM activity. PM activity was detected in four of ten progeny from plants representing callus line R18 and six of six progeny from S plants representing callus line R9.

=ryo Rescue In cases Were embryo rescue was required, developing embryos were excised from surface disinfected kernels 10-20 days post-pollination and cultured on medium containing MS salts, 2% sucrose and 5.5 g/1 Seakem agarose. Large embryos (W m) were germinated directly on the medium described above. Smaller embryos were cultured for approximately 1 week on the above medium containing 10-SM abscisic acid and transferred to hormone- free medium for germination. Several embryos became bacterially contaminated; these embryos were transferred to medium containing 300 gglml cefoxitin. Developing plants were subsequently handled as described for regeneration of S plants.

Example 11: EQrV0 Rescue

Viable progeny, recovered from seven SC82 E2/E5 plants and one SC82 E10 plant, were sustained by embryo rescue. This method consisted of excising embryos from kernels that developed on Ro plants. Embryos ranged in size from about 0.5 to 4 mm in length. Small embryos were cultured on maturation medium containing abscisic acid while larger embryos were cultured directly on gemination medium. Two of the approximately forty viable progeny thus far recovered from SC82 0 plants are shown in Figure 11B.

M. Ph"ol"e of Tranggenic Plants Most-of the RO plants regenerated from SC82 It transforma" exhibited an A188 X B73 hybrid phenotype.

Plants were similar in height to seed derived A188 plants (3-5 feet) but had B73 traits such as anthocyanin acc umu lation in stalks and prop roots, and the presence of upright leaves. Many plants, regardless of the callus line from which they were regenerated, exhibited phenotypic abnormalities including leaf splitting, forked leaves, multiple ears per node, and coarse silk.

Although many of.the phenotypic characteristics were common to all % plants, some characteristics were unique to plants regenerated from specific callus lines. Such characteristics were exhibited regardless of regeneration route and the time spent in culture during regeneration.

Nontransformed control plants were not regenerated from this culture and, therefore, cannot be compared phenotypically. Pistillate flowers developed on tassels of on, Ell (1/6), several E10 (3/22) and almost one-third of the E2/E5.(12/37) plants with a range of three to approximately twenty ovules per tassel. Primary and secondary ears developed frequently on most E2/E5, E10, and Ell plaMs; a mature E2/E5 plant is shown in Figure IIA. Anthers rarely extruded from Me tassels of plants regenerated from SC82 transformants and the limited number of a"ers which were extruded did not dehisce pollen. Som -

.ke phenotypic characteristics observed were mine to p*nts regenerated from a specific callus line such as Me lack of ears on E3/E4/E6 plants and a "grassy" phenotype (up to 21 lone narrow leaves) exhibited by all:Ell plants.

All SC82 plants senesced prematurely; leaf necrosis began approximately two weeks after anthesis. The RO plants regenerated from SC82 transformed cell lines have tended to senesce prematurely; typically before the developing kernels were mature. This has necessitated the use of embryo rescue to recover progeny (R, generation). Segregation of bar in the R, generation does not correlate with the variability in phenotypic characteristics of R, plants such as plant height and tassel morphology. In Figure 11B, the plant on the right contains bar, the plant on the left does not. In addition, most of the R, plants are more vigorous than the % plants. Transformed progeny (R1) have now also begun to yield kernels and R2 plantlets have been recovered.

Of 219 plants regenerated from 10 independent SC716 transformants, approximately 35 have reached maturity (Table 5). The SC716 plants did not exhibit the phenotypic differences which characterized the individual callus lines of SC82. These plants were more uniform and abnormalities less frequent. The phenotype of these plants closely resembled that of control plants regenerated from a SC716 cryopreserved culture which was not bombarded. Plant height ranged from three to six feet with the majority of the plants between five and six feet. Most mature plants produced large, multi-branched tassels and primary and secondary ears. Pistillate flowers also developed on tassels of several SC716 plants. Although anther extrusion occurred at approximately the same low frequency as in the SC82 plants, a small mount of pollen dehisced from some extruded anthers. For most of the SC716 plants that reached maturity, senescence did not commence until at least 30 days after anthesis. The improved characteristics of SC716 plants over SC82 plants indicate that differences between the suspension cultures may be responsible.

Mile We invention is susceptible to various modifications and alternative forms, specific emb odiments thereof have been show by way of example in the drawings and hwein be descrifed in detail. It should be understood,,however, Mat it is not intended to limit the inventiqn.t6 the particular forms disclosed, but on the contrary,. the intention is to cover all modifications, equivale"; and alternatives falling within the spirit and scopWpf the invention as defined by the appended

Claims

CLAIMS f, REFERENCES The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

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I

"T IS CLAIMED IS:

A method for the production of fertile, transgenic monocotyledonous plants, said method comprising the steps of: (a) preparing a DM composition, a component of which one desires to have expressed in, or introduced into the genome of, a monocotyledonous plant; (b) contacting recipient monocotyledonous cells with said DM composition under conditions allowing the uptake of DM by recipient cells; (c) regenerating plants from recipient cells which have received the D% component; and (e) identifying fertile, transgenic plants which contain the desired DM component.

2. The method of claim 1, wherein said monocotyledonous cells comprise maize, rice, Meat, oat, or barley cells.

3. The method of claim 1, wherein said recipient cells comprise callus cells, gametic cells, or meristematic cells.

4. The method of claim 1, wherein said recipient cells comprise cells obtained from suspension culture.

5. The method of claim 4, wherein said suspension culture is prepared from an embryogenic callus.

6. The method of claim 5, wherein said callus comprises a Me II callus.

X 7. The nethod;of claim 1, further comprising the step of pr"ariilc~ a.-.plant cell composition which includes recipient m 1 1M, prior to contacting said recipient cells with said composition.

8. The me'~hod7,;-of claim 7, wherein the plant cell compmition~ , is prepared by:

preparing an embryogenic callus; and

. 0 - (b) selecting cells from said callus, which cells comprise recipient cells.

9. The method,of claim 8, further comprising culturing Me selected callus cells in suspension culture prior to contacting with Me D% composition.

10. The method of claim 7, wherein said plant cell composition has been subjected to cryopreservation prior to contacting with Me DM composition.

11. The method.of claim 1, wherein the D= composition c compreses a transposable element.

12. The method of claim 11, wherein the transposable W 91/02071 PC'IUUS90/04462 element comprises an Ac, Ds or W element.

13. The method of claim 1, wherein the = composition comprises an exogenous gene.

4 14. The method of claim 13, wherein the exogenous gene comprises a selectable or screenable marker gene.

15. The method of claim 14, wherein the selectable or screenable marker gene comprises a neo gene, a GUS gene, a bar gene, a mutant EPSP synthase gene, a **nitrilase (^)** gene, an acetolactate synthase gene, or a gene from the R complex.

16. The method of claim 14, wherein the exogenous gene comprises a maize gene.

17. The method of claim 13, wherein the exogenous gene comprises a gene encoding a desired trait.

18. The method of claim 17, wherein the exogenous gene comprises a herbicide resistance gene, an insect resistance gene, an antifreeze protein gene, or an antifungal gene.

19. The method of claim 1, wherein the D% composition 1 comprises plasmids.

3-5 20. The method of claim 13, wherein the DM composition comprises a promoter and 31 region operatively linked to said exogenous gene.

21. Me method of claim 20, wherein said promoter comprises a CaW 35S, CMV 19S, nos, AM, sucrose synthase, R-alelle or root cell promoter.

22. The method of claim 13, wherein Me recipient cells are cotransformed with more than one exogenous gene.

23. the method of claim 22, wherein at least two exogenous genes are positioned on the same DNA

segment, and recipient cells are contacted with said segment.

24. -The method of claim 1, wherein recipient cells which have received a component are selected for prior to regeneration.

I 25. The method of claim 24, wherein cells are selected by incubation in contact with a selective medium.

26. Me method of claim 25, wherein recipient cells are contacted with a DNA composition which includes a bialaphos resistance gene, and cells which have received Me bialaphos resistance gene are selected for on a selective medium which comprises bialaphos or PPT.

27. The method of claim 1, wherein the recipient cells are contacted with Me D= composition %rough the use (^) of microprojectile'bombardment.

28. The method of claim 27, wherein uptake of the DM composition by recipient cells is achieved by microprojectile bombardment of the cells, by passing particles on which the DM composition has been coated through a screen and into the cells.

29. The method of claim 28, wherein the particles comprise tungsten or platinum.

30. The method of claim 1, wherein the recipient cells comprise embryogenic maize cells.

31. The method of claim 1. wherein the fertile.

transgenic plants comprise fertile, transgenic maize plants.

32. The method of claim 1, wherein regenerating plants from recipient cells comprises the steps of:

(a) culturing recipient cells which have received DM in a media comprising an embryogenic promoting hormone until callus organization is observed;

(b) transferring said cells to a media which includes a tissue organization promoting hormone; (c) subculturing said cells onto media without said hormones, to allow for shoot elongation or root development; and (d) transferring said cells onto a minimal medium, to provide for hardening of the plant.

3 3. The method of claim 32, wherein the embryogenic promoting hAmone.comprises dicamba.

34. Me method of claim 32, wherein the embryogenic promoting hormone comprises 2,4-D.

35. The method of claim 32, wherein the tissue organization promoting medium comprises BM, Moinositol and 2, 4 -D.

36. The mat;hod of claim 32, wherein We tissue organization promoting medium comprises ABA, BAP, IM, 2-IP or myo-inositol.

37. The method of claim 32, wherein the media employed for step (c) includes Woinositol.

1 38. The m eth od of claim 32, further comprising applying to said cells a hormone to stimulate rooting.

39. Me method of claim 38, wherein IBA is applied to stimulate rooting.

40. Me method of claim 32, wherein Me minimal media comprises Clark's media.

41. A method of producing transgenic maize plants comprising the steps of:

v (a) growing embryogenic maize cells in a suspension culture comprising media with one or more hormones; (b) preparing DM segments encoding a desired gene; (c) introducing said D% segments into said embryogenic recipient maize cells; and Q regenerating plants from said recipient maize cells.

42. Me method of claim 41, wherein said plants are fertile.

43. A fertile transgenic maize plant.

44. Me fertile transgenic maize plant of claim 43, further defined as capable of expressing an exogenous gene.

45. The fertile transgenic maize plant of claim 44, wherein said exogenous gene comprises a gene encoding a desired trait.

46. Me fertile transgenic maize plant of claim 44, wherein the exogenous gene comprises a selectable or screenable mArker gene.

i 47. A transgenic plant prepared in accordance with any one of claims 1 through 42.

48. ProgM of Me plant of claim 43.

1 ~i 49. Seeds obtained from the plant of claim 48.

50. Cells obtained from the plant of claim 43.

51. A method for providing a controlled release of a selected agent to a plant cell culture, the method comprAing Me steps of:

(a) preparing a controlled release matrix having a selected agent incorporated therein; (b) culturing plant cells in or on a desired medium to prepare a plant cell culture; and (g) placing Me controlled release matrix containing the selected agent in contact with Me medium so as to allow release of the agent into the medium.

52. The method of claim 51, wherein the selected agent comprises a na"A or synthetic plant hormone, or analog thereof.

53. The method of claim 51, wherein the plant hormone comprises 2,4-D or ".

54. The method of claim 51, wherein the controlled release matrix comprises a selected agent embedded within a silicone matrix.

55. The method of claim 54, wherein the silicone matrix comprises room temperature vulcanizing silicone.

56. The method of claim 51, wherein the controlled release matrix is dispersed within an outer tubing to form a controlled release capsule.

57. The method of claim 56, wherein at least a portion of the outer tubing is permeable to the selected

agent.

58. The method of claim 57, wherein the permeable tubing comprises a silicon-based tubing.

59. The method of claim 58, wherein the outer tubing comprises openings which have been sealed with silicone.

60. The method of claim 51, wherein the controlled release matrix has been sterilized prior to introduction into the medium.